

SHORT-TERM REGULATION OF ADIPOSE TISSUE LIPOLYSIS

A study on the actions of
some lipolytic and anti-lipolytic hormones
and drugs in isolated adipocytes

Per Björgell



Lund 1984



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and drugs in isolated adipocytes

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Title and subtitle Short-term regulation of adipose tissue lipolysis. A study on the actions of some lipolytic and anti-lipolytic hormones and drugs in isolated adipocytes.		
Abstract A modified collagenase digestion procedure for the preparation of hamster adipocytes, with high sensitivity to lipolytic and anti-lipolytic agents, was developed. Using these fat cells the anti-lipolytic effect was shown to be much more sensitive to insulin concentration than glucose uptake and utilization. The lipolytic β-adrenoceptors were also characterized. Both β_1- and β_2-receptor stimulated lipolysis was found, with a predominance of the response to β_2-adrenergic stimulation. ACTH, glucagon and two hog pituitary lipolytic polypeptide fractions were found to stimulate lipolysis in rat adipocytes through cyclic AMP-mediated phosphorylation of the regulatory phosphorylation site in hormone-sensitive lipase (HSL), with subsequent enhancement of the activity of the enzyme. Growth hormone (GH) exerts anti-lipolytic, insulin-like effects under certain conditions. Preincubation for 3 h rendered rat fat cells sensitive to the anti-lipolytic action of GH. The hormone caused this effect by a net decrease of the noradrenaline-stimulated phosphorylation and thereby reduction of the activity of HSL, notably similar in several aspects to the corresponding effect of insulin. This is the first observation that growth hormone can modulate the activity of a target enzyme by covalent modification, in the form of a phosphorylation change. Nicotinic acid was found to decrease noradrenaline-stimulated lipolysis in rat adipocytes without a decreased phosphorylation of HSL. This effect was therefore apparently unrelated to changes of cellular cyclic AMP levels. No direct effect on the HSL-activity by the drug was found. It is suggested that the observed action of the drug might have been mediated through changes of the intracellular redox state, leading to oxidation of functional SH-groups on the lipase, supported by the finding that ascorbic acid could prevent the action of the drug.		
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Date 1984-11-03

From the Department of Physiological Chemistry,
University of Lund, Sweden

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Till

Astrid, Anna och Rasmus

"Allt är ju ändå som det **kan** vara.
Vore det annorlunda då vore det
också som det då **kunde** vara,
men inte annorlunda än det **kunde**."

Vägdamm

i Vägen till Klockrike

Harry Martinsson

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LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals.

- I. Björgell, P., Nilsson, N.O. and Belfrage, P.
Effects of insulin on lipolysis and lipogenesis in hamster white adipocytes with high sensitivity to hormones.
Biochim. Biophys. Acta 1981, 666, 246-251
- II. Björgell, P. and Belfrage, P.
Characteristics of the lipolytic beta-adrenergic receptors in hamster adipocytes.
Biochim. Biophys. Acta 1982, 713, 80-85.
- III. Björgell, P., Trah, T., Schleyer, M. and Belfrage, P.
Activation of adipose tissue lipolysis by ACTH, glucagon and hog pituitary lipolytic peptides through phosphorylation of hormone-sensitive lipase.
In manuscript. 1984.
- IV. Björgell, P., Rosberg, S., Isaksson, O. and Belfrage, P.
The anti-lipolytic, insulin-like effect of growth hormone is caused by a net decrease of hormone-sensitive lipase phosphorylation.
Endocrinology 1984, 115, 1151-1156
- V. Björgell, P. and Belfrage, P.
The anti-lipolytic effect of nicotinic acid: Inhibition of lipolysis in rat adipocytes without change of the phosphorylation of hormone-sensitive lipase.
In manuscript. 1984.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
pACTH	porcine ACTH
BSA	bovine serum albumin
cAMP-PrK	cyclic AMP dependent protein kinase
cyclic AMP	cyclic adenosine 3',5'-monophosphate
DFP	diisopropyl fluorophosphate
ED ₅₀	50% of the maximally stimulatory concentration
FFA	free fatty acids
GH	human growth hormone
HEPES	2-N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSL	hormone-sensitive lipase
IC ₅₀	50% of the maximally inhibitory concentration
MSH	melanocyte-stimulating hormone
PCV	packed cell volume
PLF	porcine lipolytic fraction
PrP	protein phosphatase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Staph. aur.	Staphylococcus aureus
TSH	thyroid-stimulating hormone

INTRODUCTION

The adipose tissue is mainly composed of fat cells, adipocytes. Each fat cell consists of a large fat-globule, representing about 90% of the mass, surrounded by a thin rim of cytoplasm and a protruding nucleus. The fat stored as triacylglycerols makes up 10-15% of the human body weight and provides a highly concentrated form of energy. Regarded as an organ, the adipose tissue is one of the largest in the body. The adipose tissue fat is quantitatively the most important source of fuel in mammals.

The flow of fat from and to the adipose tissue depends on the state of nutrition and energy requirements of the body. Through lipogenesis, stimulated after a meal, triacylglycerols are formed and deposited in the adipocytes, as are the chylomicron triacylglycerols. Fatty acids and glycerol are, when needed, released to the blood by hydrolysis of the fat, in the process of lipolysis. This is a result of sympathetic and/or hormonal activation. Fast-acting lipolytic hormones such as adrenaline, noradrenaline, ACTH and glucagon increase the rate of lipolysis, whereas insulin, for example, leads to the opposite, an anti-lipolytic and lipogenetic effect.

The adipose tissue lipolysis, in the following referred to as lipolysis, is regulated by the activity of hormone-sensitive lipase (HSL), which catalyzes the rate-limiting, first step in the hydrolysis of triacylglycerols. As a result monoacylglycerols will be formed, which are then mainly hydrolyzed by a specific monoacylglycerol lipase (Tornqvist and Belfrage, 1976) to glycerol and fatty acids.

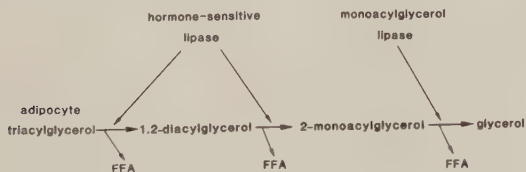


Fig. 1. Main reaction sequence of adipose tissue lipolysis.



This thesis deals with the short-term regulation of lipolysis, specifically how certain fast-acting lipolytic hormones and drugs affect the extent of phosphorylation and/or activity of HSL in isolated adipocytes.

BACKGROUND

Free fatty acids (FFA)

The free fatty acids (FFA) are one of the major energy resources and represent the quantitatively most important fuel in mammals. The fatty acids released are transported in the blood as albumin-bound FFA, as first suggested by Laurell (1956). The molar ratio FFA:albumin rarely exceeds 2:1 in the blood (Scow and Chernick, 1970; Spector and Fletcher, 1978). However, higher ratios could be expected in the vicinity of cells, especially at the surface of the adipocytes, in view of the high turn-over rate of the plasma FFA, $t_{1/2}=1-2$ min (Havel, 1965; Scow and Chernick, 1970; Davies and Souness, 1981).

Clinically, excessive release of FFA may be injurious in several respects, particularly when the molar FFA:albumin ratio is high, more than 2:1, as discussed by Spector and Fletcher (1978). Excessive FFA release may lead to pathological hepatic triacylglycerol deposition and production of very low density lipoproteins and ketone bodies in the liver, which may be contributing factors in atherogenesis (Steinberg, 1972). Also, an association between elevated serum FFA levels and serious cardiac arrhythmias has been reported in patients with acute myocardial infarction (Oliver et al., 1968). Inhibition of lipolysis reduces the extent and severity of experimentally induced myocardial ischemic injuries (Kjekshus and Mjøs, 1973). The mode of regulation of the FFA release is thus of clinical importance, and therefore also the modulation of the lipolytic process by drugs and hormones.

Hormone-sensitive lipase (HSL)

The existence of a hormone-stimulated activity in adipose tissue extracts was described already in the early 1960s (Hollenberg et al., 1961; Rizack, 1961; Björntorp and Furman, 1962), and Vaughan et al. (1964) assigned it the term "hormone-sensitive lipase". The

enzyme has been extraordinarily difficult to isolate. However, as recently reported, it has now been detergent-solubilized, extensively purified and partially characterized (Fredrikson et al., 1981). HSL subunits have an apparent minimum M_r of 84000, as determined by SDS-PAGE, and a pI of 6.8 (4°C). The enzyme hydrolyzes tri-1,2-di-, and 2-monoacylglycerol:cholesterol ester at the maximal relative rates of 1:10:1:1.5 (Fredrikson et al., 1981; Fredrikson and Belfrage, 1983). It preferentially attacks the 1(3)-bonds, however, the preference is less marked than that of e.g. pancreatic and lipoprotein lipase. Diisopropyl fluorophosphate (DFP) and cysteine-directed reagents (Hg^{2+} , N-ethylmaleimide) inhibit the HSL, indicating a reactive serine residue in the catalytic site and one or several functional SH-groups (for review, see Belfrage et al., 1984). Although the adipose tissue HSL concentration is low, about 2 μg enzyme protein/g tissue, the enzyme constitutes a large lipolytic capacity due to its high specific enzymatic activity (Fredrikson et al., 1981; Strålfors and Belfrage, 1983a).

Regulation of hormone-sensitive lipase

Hormone-sensitive lipase is rapidly phosphorylated and thereby activated by cyclic AMP-dependent protein kinase (cAMP-PrK) (Strålfors and Belfrage, 1984). HSL is phosphorylated in intact fat cells from rat as shown by the isolation of ^{32}P -labelled enzyme protein from adipocytes. Preincubation with [^{32}P]orthophosphate labels the intracellular ATP, the terminal phosphate of which, after hormonal stimulation, is incorporated into the lipase (Belfrage et al., 1980; Nilsson et al., 1980; Björgell et al., IV; for recent reviews, see Belfrage et al., 1984; Strålfors and Belfrage, 1984). Norepinephrine enhances cellular levels of cyclic AMP, cAMP-PrK activity and HSL phosphorylation and activity in a dose dependent manner (Nilsson et al., 1980; Nilsson, 1981). These results establish the "lipolytic activation cascade" essentially as originally proposed by Steinberg and Huttunen (1972). The HSL phosphorylation takes place on serine residues at two different phosphorylation sites (Strålfors et al., 1984). The phosphorylation of the "basal" site is not affected by hormones, whereas the second "regulatory" site is identical to that phosphorylated by cAMP-PrK in vitro. This has been shown by two-

dimensional peptide mapping of phosphopeptides from proteolytically degraded ^{32}P -HSL from isolated adipocytes (Strålfors et al., 1984). The rate of phosphorylation of the isolated lipase is comparable to that in fat cells. As this phosphorylation is promptly arrested by the addition of the specific inhibitor protein of cAMP-PrK the possibility of an intervening lipase kinase, analogous to phosphorylase kinase, is excluded (Fredrikson et al., 1981).

The phosphorylation of HSL is reversible. Partially purified protein phosphatases (PrP) from rat adipose tissue (Olsson et al., 1984) and rabbit muscle PrP type 1, 2A and 2C (Olsson et al., unpublished), dephosphorylate and deactivate the enzyme. Recent findings indicate that type 2A predominates the PrP activity against HSL in rat adipose tissue (H. Olsson, personal communication).

Insulin added to noradrenaline-stimulated fat cells rapidly decreases the extent of HSL phosphorylation and activity (Nilsson et al., 1980; Nilsson, 1981). The decreased phosphorylation state reflects a net dephosphorylation of the regulatory site (Strålfors et al., 1984). The mechanism through which insulin causes this net dephosphorylation of the HSL regulatory site has not been established. A decreased cAMP-PrK activity and/or an increased PrP activity may mediate the net dephosphorylation of HSL. Insulin decreases the levels of cyclic AMP endogenously bound to protein kinase, as well as the activity of cAMP-PrK in noradrenaline-stimulated adipocytes (P. Björgell, unpublished). Preincubation of fat cells with insulin prior to noradrenaline stimulation substantially reduces the cellular cyclic AMP levels induced (Nilsson, 1981). The anti-lipolytic effect of insulin thus certainly involves, but to an unknown extent, cyclic AMP reduction, decreased cAMP-PrK activity and a decreased rate of phosphorylation of HSL.

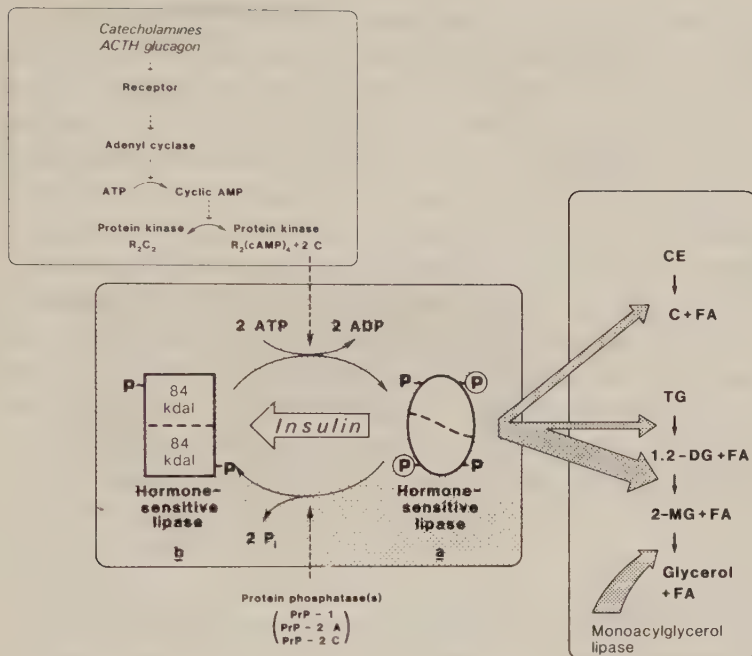


Fig. 2. Hormonal control of adipose tissue lipolysis through reversible phosphorylation of hormone-sensitive lipase. Arrow, with insulin, indicates the net dephosphorylation of the enzyme, accounting for insulin's anti-lipolytic effect. The dimeric structure of the enzyme is tentative. Encircled -P, phosphorylation of regulatory sites; CE, cholesterol ester; TG, DG, MG, tri-, di- and monoacylglycerol; FA, fatty acid.

The present work represents one part of a long-term project concerning the hormonal and pharmacological control of adipose tissue lipolysis. The background and results of the respective manuscripts will be discussed separately.

GENERAL METHODOLOGY

Preparation and incubation of adipocytes (I-V)

In 1964 Rodbell described a method for preparation of rat adipocytes by collagenase digestion (Rodbell, 1964). By the use of this technique homogenous cell preparations are obtained, diffusion problems reduced and the viability of the cells controlled. The condi-

tions differ from those in intact adipose tissue, but basal mechanisms for drug and hormonal action are likely to be the same. The fat cells are, for example, deprived of their sympathetic innervation and they are incubated in an accumulating system. This has to be observed, with regard to e.g. local FFA (Fain and Shephard, 1975) and adenosine concentrations (Hjemdahl and Fredholm, 1976a; Fredholm and Hjemdahl, 1979) when discussing the results. The rat adipocytes used in this study have a high sensitivity to hormones, as exemplified by the ED_{50} (concentration giving 50% of maximally stimulatory effect) of 40 nM for noradrenaline stimulated lipolysis and IC_{50} (concentration giving 50% of maximally inhibitory effect) of 30 pM for the anti-lipolytic effect of insulin after 200 nM noradrenaline stimulation (cf. Nilsson and Belfrage 1979, 1981; Nilsson et al. 1980; Nilsson, 1981; Björgell et al., 1984 (IV); Strålfors et al., 1984).

The rat adipocytes, which have been used in papers III-V, were prepared as previously described (Nilsson and Belfrage, 1978, 1981) by collagenase digestion. Hamster adipocytes have been used in papers I and II. Catecholamines activate lipolysis by the binding to and stimulation of β -adrenoceptors. The classification of these receptors is controversial. For studying the lipolytic β -adrenoceptors in adipose tissue (II), fat cells from the golden hamster (*Mesocricetus auratus*) were chosen, since such adipocytes, like human, were reported to have both α - and β -adrenoceptors and a range of hormonal responses, similar to those found with human adipose tissue (Hittelman et al., 1973; Rosak and Hittelman, 1977). However, the hormonal sensitivity of the fat cells, prepared according to the protocol routinely used (Hittelman et al., 1973), was found to be low (I). Fine mincing of the fat pads, followed by a short (5 min) and vigorous shaking of the tissue in a buffer with high collagenase concentration (1.67 mg/ml), in our hands usually resulted in damaged adipocytes with low sensitivity to lipolytic hormones. Also, as originally reported (Rosak and Hittelman, 1977) noradrenaline-stimulated lipolysis was quite insensitive to insulin inhibition in fat cells prepared in this way. We therefore developed a modified preparation procedure as described in paper I, which was sub-

sequently used in paper II, and also by other workers (e.g. Schimmel, 1984). The hamster adipocytes, prepared as described (I), were 4-8-fold more sensitive to catecholamines and also more sensitive to the anti-lipolytic action of insulin than cells prepared by the method of Hittelman et al. (1973) routinely employed in previous studies of lipolysis in hamster fat cells.

As detailed in paper I some important factors for successful preparations were the use of young hamsters, reciprocal shaking and low concentration of collagenase. Hamster adipose tissue was more sensitive than that of the rat to collagenase. It was also of importance to select an appropriate collagenase batch, since the collagenase preparations used contain clostripain and other unspecific proteases. The fat pads had to be carefully handled and not minced. Optimal cell quality was obtained before the fat pads were completely disintegrated.

The intactness of the cells in the present studies (I-V) have routinely been evaluated by the ability of insulin to stimulate the conversion of [3-³H]glucose to cell lipids (Moody et al., 1974), as described in the various papers.

Determination of the lipolysis rate

FFA release rate was measured continuously as the proton release rate, which is equivalent to FFA release rate (Rudman and Shank, 1966; Nilsson and Belfrage, 1979), by the use of a pH-stat titration system (cf. Nilsson and Belfrage, 1979, 1981). With two parallel pH-stat titrators (as in papers IV and V) controls and experiments with added agonists or antagonists could be performed simultaneously. The pH-stat titration technique is an invaluable tool for monitoring the FFA release during short-term studies of hormonally induced lipolysis, allowing cell samples for biochemical parameters, such as the extent of HSL phosphorylation, to be taken at precisely the correct time-points.

The "lipolysis rate" is generally measured by the glycerol release rate, since little glycerol is re-utilized in the fat cells. However, also the FFA release rate reflects lipolysis under the

conditions used in the experiments (high BSA/FFA molar ratios, short incubation times, relatively dilute cell suspensions) (cf. Rodbell, 1965), since the reesterification of released fatty acids is insignificant (Fig. 3, cf. also Nilsson and Belfrage, 1979; Nilsson et al., 1980; Nilsson, 1981).

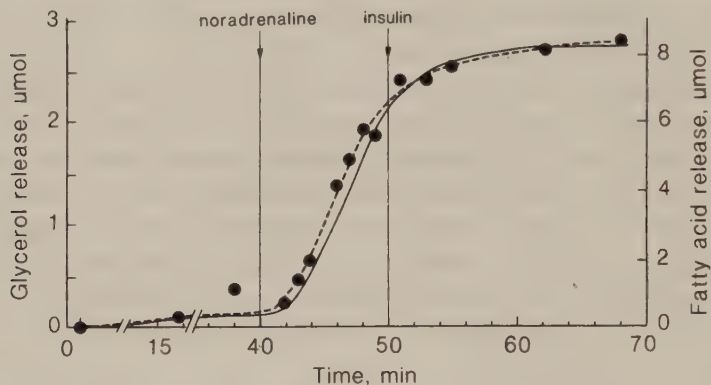


Fig. 3. Relationship between the release of fatty acids and glycerol from fat cells. Rat adipocytes (50 μ l PCV/ml) were incubated in 10 ml of modified Krebs-Ringer solution with 3.5% (w/v) bovine serum albumin as detailed in the Materials and Methods section of papers III-V. Release of fatty acids was monitored continuously by pH-stat titration (continuous line). Glycerol was determined in samples (duplicates) from the same incubation. At the indicated time-points adipocytes were exposed to 200 nM noradrenaline and 700 pM insulin. The values for fatty acid release have been corrected for the volume change caused by glycerol sampling (P. Björgeell, unpublished).

Thus, FFA release paralleled the glycerol release at an approx. 3:1 molar ratio both after exposure of the cells to noradrenaline and to noradrenaline plus insulin (Fig. 3). Therefore, the lipolysis rate will be considered to be reflected by both parameters in this study and, in turn, to be a measure of the activity of the HSL in situ, in the intact adipocytes.

The enzymatic fluorometric method has been used for the glycerol determinations (Laurell and Tibbling, 1966; Nilsson and Belfrage, 1978).

Determination of the extent of phosphorylation of HSL in intact adipocytes

This procedure, as described by Belfrage et al. (1980) and Nilsson et al. (1980), makes it possible to follow a covalent modification of HSL, the rate-limiting enzyme in the hydrolysis of fat, in parallel with its biological activity, measured as the FFA release rate. In brief, adipocytes are incubated with [^{32}P]ortho-phosphate which labels the intracellular ATP pool to a constant specific activity. HSL is labelled on a basal serine site (cf. Strålfors et al., 1984) over a 40 min period and then, after hormonal stimulation, the HSL is phosphorylated on the regulatory serine site (cf. Strålfors et al., 1984). The extent of HSL phosphorylation was estimated from the ^{32}P -content of the $M_r=84000$ phosphoprotein band in autoradiographs after SDS-PAGE of delipidated whole-cell proteins. This band has been identified with ^{32}P -HSL (Belfrage et al., 1980; Nilsson et al., 1980) as further evidenced by the experiments described in Fig. 4 (P. Strålfors, unpublished). The pattern of proteolytic ^{32}P -phosphopeptides, obtained after Staph. aur. V8 protease digestion of the $M_r=84000$ phosphoprotein obtained directly by SDS-PAGE of the fat cell proteins was closely similar to the pattern obtained from homogenous ^{32}P -labelled HSL isolated from intact adipocytes after several chromatographic steps.

Since the original reports (see above) some smaller modifications have been introduced and employed throughout this study (III-V). Protease inhibitors have been added to the cell-disrupting SDS-solution (10 $\mu\text{g}/\text{ml}$ of each of leupeptin, antipain and pepstatin), also in paper IV (in which this information has been left out by mistake). The gels were autoradiographed without intensifying screens (see Nilsson et al., 1980) to get less dispersion and thus sharper ^{32}P -phosphopeptide bands on the films. The relative ^{32}P -content was estimated as the peak height of the ^{32}P -HSL band (cf. Nilsson et al., 1980; Björgell et al., paper IV), which correlated well with ^{32}P -radioactivity determined by scintillation counting of cut-out and dissolved gel-slices (Nilsson et al., 1980). Base line positioning and calculations of peak heights on the scanning den-

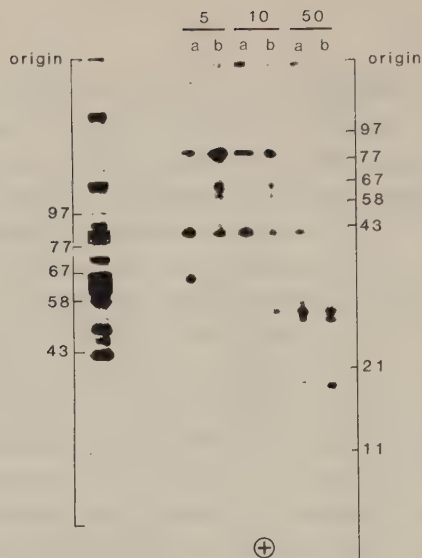


Fig. 4. Comparison between the ^{32}P -phosphopeptides from proteolytically degraded $M_r=84000$ ^{32}P -phosphoprotein obtained by SDS-PAGE of whole fat cell protein extracts, and from ^{32}P -labelled HSL, isolated from intact fat-cells. Adipocytes from 50 rats were incubated as described in the Materials and Methods section in papers III-V except that a $100\ \mu\text{l}$ PCV/ml cell suspension containing $50\ \text{mCi } ^{32}\text{P}_i$ was used. After 50 min preincubation the cells were exposed to $10\ \text{ng/ml ACTH}_{1-24}$ and further incubated for 5 min. Samples were either withdrawn for SDS-PAGE or homogenised in $0.25\ \text{M}$ sucrose, $1\ \text{mM}$ dithioerythritol, $1\ \text{mM}$ EDTA, $2\ \text{mM}$ ATP, $5\ \mu\text{g/ml}$ of the protease inhibitors leupeptin, pepstatin and antipain, $10\ \text{ng/ml}$ ACTH and $20\ \text{mM}$ NaF at pH 7.4. HSL was purified from the homogenate (Belfrage et al., 1980) up to and including the gradient-sieve/porpive chromatography and hydroxylapatite chromatography. Enzyme-containing fractions were pooled, the protein precipitated with 5% (w/v) trichloroacetic acid in 30% (v/v) acetone, with fragmented DNA as carrier, and subjected to SDS-PAGE (Strålfors and Belfrage, 1983). Gel slices containing ^{32}P -labelled HSL were excised after the SDS-PAGE of whole-cell proteins (the $M_r=84000$ ^{32}P -phosphoprotein band indicated on the autoradiography to the left in the Figure) and of the purified lipase, respectively. The gel slices were treated for and subjected to limited proteolysis and peptide mapping as described in Cleveland et al. (1977). After SDS-PAGE in a linear polyacrylamide-gel gradient ($T=10-22\%$, $C=0.9\%$) the gel was stained, dried and autoradiographed. a ^{32}P -phosphopeptides from the pure HSL; b from the $M_r=84000$ ^{32}P -phosphoprotein band of whole-cell proteins; digestion with 5, 10 or 50 ng Staph. aur. V8 protease, as indicated. Molecular weight markers used were: glycogen phosphorylase (97,400), transferrin (75,700), bovine serum albumin (67,000), catalase (57,500), ovalbumin (43,000), soybean trypsin inhibitor (21,000) and pancreatic colipase (11,000), the molecular weights ($\times 10^{-3}$) are indicated. By courtesy of P. Strålfors (unpublished).

sitograms were performed essentially as described by Yakin et al. (1982).

EFFECTS OF INSULIN ON LIPOLYSIS AND LIPOGENESIS IN HAMSTER ADIPOCYTES (I)

Inhibition of hormone-induced lipolysis and enhanced glucose transport are the two major insulin effects in fat cells. The sensitivity of these two processes to insulin concentration is a matter of controversy. It has been reported that the anti-lipolytic effect is more sensitive to insulin than is the glucose uptake (Fain et al., 1966; Hepp et al., 1967). Other reports, however, have indicated that insulin affects both processes over the same concentration range (Green and Newsholme, 1979; Thomas et al., 1979). In paper I the effects of varying insulin concentrations on lipogenesis, measured as [3-³H]glucose conversion to cell lipids (Moody et al., 1974), and on catecholamine-stimulated lipolysis have been compared under identical conditions with the same batches of highly sensitive hamster adipocytes. Lipogenesis, measured under the experimental conditions used, is mainly controlled by the rate of glucose uptake (Gliemann et al., 1984). The measured parameter thus reflects the glucose uptake. The anti-lipolytic effect of insulin was dose-dependent with an IC₅₀ at approx. 40 pM insulin after stimulation with catecholamines. ED₅₀ for lipogenesis after incubation with [3-³H]glucose and insulin was approx. 250-300 pM. Thus, there was an almost 8-fold difference in insulin sensitivity, in accordance with the results of Nilsson (1981), who found, in rat adipocytes, an IC₅₀ for the anti-lipolytic effect of insulin on noradrenaline-stimulated lipolysis of 20-30 pM and an ED₅₀ of 80 pM for lipogenesis after incubation with [3-³H]glucose, noradrenaline and insulin under the same conditions.

A recent report has shown that incubations with noradrenaline inhibited insulin-stimulated glucose transport in rat adipocytes (Kirsch et al., 1983). We have, however, not been able to confirm this effect in similar studies of the insulin sensitivity of lipogenesis in rat adipocytes (P. Björgell, unpublished). Furthermore, lipogenesis measured as the conversion of [3-³H]glucose to cell

lipids, after incubation with insulin, was not significantly affected by the addition of isoprenaline to the medium (cf. Figs. 1b and 3 in paper I). It can be concluded that, if determined under identical experimental conditions, the sensitivity of the antilipolytic action to insulin is considerably higher than that of a parameter reflecting glucose uptake. The results imply that the mechanisms whereby insulin affects the two processes differ.

BETA-ADRENOCEPTORS IN HAMSTER ADIPOCYTES (II)

The most important fast-acting hormones stimulating the adipose tissue lipolysis are the circulating (noradrenaline and adrenaline) and neural (noradrenaline) catecholamines (Rosell and Belfrage, 1979). They act through stimulation of adrenergic receptors. Ahlquist originally classified the adrenergic receptors in a variety of organs as α - and β -receptors (Ahlquist, 1948). In 1967 Lands et al. subdivided the β -adrenoceptors into β_1 - and β_2 -receptors by the use of sympathomimetic amines (Lands et al., 1967). The adipocyte β -adrenoceptors were, like the cardiac receptors, classified as β_1 . Most studies in mammals suggest that there are only two subtypes of β -adrenoceptors (Nahorski, 1981). However, the classification of lipolytic β -adrenoceptors has been questioned by several groups, first by Grana et al. (1972), Stanton (1972) and Harms et al. (1974). Harms et al. (1974) evaluated the hypothesis of Lands et al. (1967) by studying the effect of several β -adrenoceptor antagonists on lipolysis induced by isoprenaline in rat adipocytes. The results obtained cast serious doubts on the β_1 -classification and it was later proposed by this group that the rat adipocyte β -receptor should be regarded as an atypical, homogeneous β -adrenoceptor with a dualistic, hybrid β_1 - and β_2 -character (Harms et al. 1974, 1975; de Vente et al., 1980). It has recently been argued (Bojanic and Nahorski, 1983) that the hypothetical, hybrid β -adrenoceptor in the rat has such a low affinity that it would probably not be detected when using high affinity ligand binding assays.

However, work by others indicated that both receptor subtypes (β_1 - and β_2 -adrenoceptors) might co-exist in the same tissue

(Carlsson et al., 1972; Furchgott 1976; Nahorski et al., 1979) and that both types could take part in the same biological response. With regard to the adipocyte β -adrenoceptors it has been discussed, with regard to different species (cf. Table I), whether there are two separate β_1 - or β_2 -adrenoceptors, co-existing in the adipose tissue (e.g. Deacon, 1978; Björgeell et al., 1982 (II); Lai et al., 1982; Bojanic and Nahorski, 1983; Hjerdahl and Linde, 1983), or if the adipocytes have only one type of β -adrenoceptor, either β_1 or β_2 depending on the species (e.g. Goldberg et al., 1975; Frisk-Holmberg and Östman, 1977; Jolly et al., 1978; Pfeile et al. 1981), or, finally, if there is a specific β -adrenoceptor in the adipose tissue with dualistic β_1 - and β_2 -characteristics (e.g. Harms et al., 1974, 1975; de Vente et al., 1980; Tan and Curtis-Prior, 1983; Mersmann 1984a,b; Wilson et al., 1984). It has been proposed that there could be two subpopulations of a β_1 -adrenoceptor in human adipose cells (Jacobsson et al., 1981).

Table I. Classifications¹⁾ of the β -adrenoceptors present in white adipose tissue of some mammals

Species	β_1 -adrenoceptors	β_2 -adrenoceptors	β_1 - and β_2 -adrenoceptors	* β_3 -adrenoceptors ²⁾
Rat	Lands et al., 1967 Belfrage and Fredholm, 1978 Cox et al., 1982	Jolly et al., 1978 Pfeile et al., 1981	Björgeell et al., unpublished Bojanic and Nahorski, 1983	Stanton, 1972 Harms et al., 1974, 1975 De Vente et al., 1980 Little and de Haen, 1980 Tan and Curtis-Prior, 1983 Wilson et al., 1984
Man	Kather and Simon, 1977 Frisk-Holmberg and Östman, 1977 Jacobsson et al., 1981	Goldberg et al., 1975 Pfeile et al., 1981	Wenkeova et al., 1976 Deacon, 1978 Hjerdahl and Linde, 1983	Harms et al., 1975 Kather and Simon, 1980 Ronn et al., 1980
Hamster			Björgeell and Belfrage, 1982 (II)	
Dog	Kelly and Shanks, 1975 Frie et al., 1979		Belfrage and Fredholm, 1978 Hjerdahl et al., 1979 Belfrage, 1978	
Mouse		Jolly et al., 1978		
Swine				Mersmann, 1984a,b
Cat	Lockwood and Lum, 1974			Loakpradit and Lockwood, 1977

¹⁾In some cases the classifications have been interpreted from the results of a particular report.

²⁾The term " β_3 " refers to β -adrenoceptors which have properties of both β_1 - and β_2 -adrenoceptors

In paper II we have examined the characteristics of the lipolytic β -adrenergic receptors in hamster adipocytes. Using lipolytic β -agonists, selective for one of the receptor types, in combination with selective β -antagonists for the other receptor type (cf. Table II) with highly sensitive adipocytes, results were obtained which

suggested that both β_1 - and β_2 -adrenoceptors were present in the fat cells. The β_2 -receptor mediated activation of lipolysis seemed to predominate, as was recently also indicated by results obtained by Hjendahl and Linde (1983) in human adipose tissue in vivo.

Table II. Effects of some β -adrenergic drugs

A. β -agonists	Adrenoceptorstimulating effects		
	α	β_1	β_2
Noradrenaline	+++	++	+
Adrenaline	+++	+	+++
Isoprenaline	(+)	+++	+++
Terbutaline		+	+++
Salbutamol		+	+++
Procaterol		(+)	+++
Prenalterol		+	
B. β -antagonists	Adrenoceptorblocking effects		
	β_1	β_2	
Propranolol	+++	+++	
Atenolol	++	(+)	
Practolol	++	(+)	
Metoprolol	++	(+)	
Pamamolol	+++	(+)	
ICI 118,551	(+)	+++	
H 35/25	(+)	++	

The different results obtained by us and others may have several explanations. First, the earliest studies were performed with the assumption that only one type of β -adrenoceptors would be found in each tissue, in line with Lands' proposal. Second, most studies had been performed as comparisons between different lipolytic and anti-lipolytic β -adrenergic drugs, but without the concomitant use of a drug which blocked the receptor type not studied. This is probably necessary as very few β -agonists and β -antagonists are sufficiently selective (cf. Table II). Third, when using adipocytes the cell preparation technique must be carefully controlled. As the collagenase preparations used contain different unspecific proteases a selective digestion of one or the other of the β -receptor subtypes may result. In our hamster adipocyte study (II) most of the β_1 -adrenergic responses (Rosak and Hittelman, 1977) disappeared when we used a harsh collagenase digestion technique (Hittelman et al., 1973). However, when using the milder digestion technique (I), both

β_1 - and β_2 -responses were present, the β_2 -receptor stimulated lipolysis predominating (the ratio β_2/β_1 was approx. 2). Another study of Bojanic and Nahorski (1983) showed 62% β_2 - and 38% β_1 -receptors in whole fat pads, but 15% β_2 - and 85% β_1 -receptors in isolated adipocytes. It was proposed that a large proportion of the β_2 -adrenoceptors were located on other cell types than fat cells. However, the results could also be explained by a proteolytic degradation of β_2 -adrenoceptors during the collagenase digestion.

Fourth, when interpreting the results of in vivo-based β -adrenoceptor classifications the influence of catecholamines on the adipose tissue blood flow has to be born in mind, as discussed by Hjendahl and Linde (1983). Differences in the adipose tissue blood flow in response to noradrenaline and adrenaline between animal species may thus contribute to the differences in relative lipolytic potency of the catecholamines reported.

In conclusion, when viewing the results of our and other groups (cf. Table I) it seems likely that both β_1 - and β_2 -adrenergic receptors are present in adipose tissue in mammals, even if one of the receptor types may predominate in different species. None of the studies examined seem to contradict this conclusion. In many reports the importance of β_2 - rather than β_1 -adrenoceptors in adipose tissue is emphasized. Interestingly, Lai et al. (1982) have reported a shift from β_1 -adrenoceptors in 3T3-L1 preadipocytes to β_2 -adrenoceptors in differentiated adipocytes, the differentiation induced by dexamethasone, a glucocorticoid analog. It has been proposed that β_1 -adrenoceptors were involved in the control of basal lipolysis in man, whereas β_2 -adrenoceptors would be more effective following stress, since lower plasma FFA concentrations were observed with propranolol, a non-specific β -antagonist, than with atenolol, a β_1 -selective antagonist (Deacon, 1978). The clinical implication of the study was that the cardioselective β_1 -adrenergic blockers may be less effective in suppressing stress-induced rises in plasma FFA.

EFFECTS OF ACTH, GLUCAGON AND HOG PITUITARY PEPTIDES ON THE ACTIVITY AND PHOSPHORYLATION OF HORMONE-SENSITIVE LIPASE (III)

It is well-known that ACTH and glucagon, as well as a number of other fast-acting lipolytic hormones, like α - and β -MSH, TSH, vasopressin and secretin stimulate lipolysis in adipocytes (for review, see Davies, 1973). Several findings indicate that the effects of ACTH and glucagon are mediated through the "lipolytic activation cascade", via adenylate cyclase activation, cyclic AMP production, cAMP-PrK activation and phosphorylation of HSL. This was, in fact, generally believed to be the activating mechanism although it had never been directly demonstrated.

As we had access to methods for examining the phosphorylation and activation of HSL in intact fat cells, one of the purposes of this work (III) was to experimentally verify or disprove the proposed mode of lipolytic action(s) of ACTH and glucagon. Through the work of Schleyer et al. (1969, 1976a,b) certain chromatographic fractions containing lipolysis activating polypeptides had been obtained from hog pituitary glands, termed PLF II C and PLF II D (PLF=Porcine Lipolytic Fractions). The mode of action of these lipolytic polypeptides was unknown. Therefore, another purpose of this study (III) was to examine this, by comparison with ACTH and glucagon. The work in paper III has been performed with ACTH¹⁻²⁴ ($M_r=2934$) and porcine glucagon ($M_r=3485$) besides the PLF II C and D. Since the work in paper III was carried out further characterization of PLF II C and D has been done by Trah (1983), which will be discussed below.

The results (III) showed that ACTH¹⁻²⁴ was the most active of the lipolytic peptides tested, with an ED_{50} of about 1 pM. The ED_{50} s, calculated from the lipolytically active peptide concentrations, for PLF II D, glucagon and PLF II C were 0.7 nM, 3 nM and 60 nM. Similar maximal rates of lipolysis were obtained with each of the lipolytic agents. Others (e.g. Oelofsen and Ramachandran, 1983; Heckemeyer et al., 1983) have found similar lipolytic potencies for ACTH and glucagon. All the polypeptides studied rapidly enhanced the HSL phosphorylation and activity. Insulin reversed the PLF II C and D

stimulated phosphorylation and activity of HSL. Propranolol had no effect on lipolysis induced by PLF II C and D.

Although the lipolytic potency of the polypeptides differed these results indicate that ACTH, glucagon and the hog pituitary polypeptides all exert their lipolytic action through cyclic AMP-dependent protein kinase catalyzed, reversible phosphorylation of the regulatory serine site on HSL (Nilsson et al., 1980; Strålfors et al., 1984).

The polypeptides in fractions PLF II C and D, which are in the M_r range of 5000-6000 by SDS-PAGE, have recently been further characterized and parts of their primary structures determined (Trah, 1983). Although not a part of the work of this thesis these results are of a certain interest with regard to the mechanism of action of these polypeptides, and will therefore briefly be discussed. One specific polypeptide, termed C3, representing about 30% of the PLF II C peptides, accounted for the lipolytic activity of this preparation. It had an M_r of about 5000 and a $pI=8.0$. Most part of the primary structure of C3 has been determined (Fig. 5).

1	5	10	15
Phe-Leu-Gly- X	-Pro-Thr- X	-Lys-Thr-Tyr-Arg-Pro-His-Phe-Asn-	
16	20	25	30
Leu-Ser-His-Gly-Lys-Asp-Gln-Val- X	-Ala-His-Gly-Gln- X	-Val-	
31	40	43	
Ala-(	32-39	)	-Val-Lys-Phe-Leu

Fig. 5. Part of the amino acid sequence of the peptide C3. From Trah (1983).

The PLF II D fraction was composed of several peptides (Trah, 1983). The lipolytic activity was mainly found in a fraction, termed D3, containing about 40% of the PLF II D peptides. D3 contained four peptides, D3₁, D3₂, D3₃ and D3₄, (separated by reversed-phase HPLC), which were all equally lipolytically active. The molecular weight was approx. 5000-6000 for each of the peptides with pI 's between 9-

10. The N-terminal parts of D₃₁₋₄ were found to be quite similar to that of porcine ACTH, (pACTH).

Further studies by Trah (1983) revealed a C-terminal difference between ACTH and the D₃ peptides. It was therefore proposed (Trah, 1983) that the PLF II D₃ subfractions could be variants of ACTH, in analogy with variants of other hormones (Larsson et al., 1979; Guillemin et al., 1982). These findings could possibly explain the high lipolytic activity of PLF II D (paper III). However, the exact relation of these lipolytically active D₃ polypeptides to pACTH and pACTH-precursors obviously remains to be established.

The lipolytically active polypeptide PLF II C 3 on the other hand showed no sequence homology with pACTH (Fig. 5). Although less active than ACTH and the D₃ peptides in rat adipocytes (III) it has been reported to have a relatively higher lipolytic potency in swine adipose tissue. It has been proposed to represent a novel lipolytic pituitary polypeptide in this species (Trah, 1983) although this naturally requires additional experimental evidence.

MODE OF ANTI-LIPOLYTIC ACTION OF GROWTH HORMONE (IV)

Growth hormone (GH) or somatotropin is secreted by the adenohypophysis every third hour as pulsatile peaks in rat plasma ranging from the basal levels of about 1 ng/ml up to 200-400 ng/ml (Tannenbaum and Martin, 1976; Edén et al., 1978). Besides the GH effect on somatic growth, the hormone has many metabolic effects. In adipose tissue GH exerts divergent effects, which may, depending on the experimental design, be classified as either anabolic (insulin-like) or catabolic. Thus, administration of GH in vivo, especially to fasted animals, leads to a decrease of plasma FFA levels, which is later followed by an increase (reviews Davies, 1973; Isaksson et al., 1984). GH is one of the slow-acting lipolytic hormones, which causes a modest increase of basal lipolysis after several hours, probably by increasing the synthesis of proteins involved in the lipolytic activation process (Fain et al., 1965; Fain, 1982; Goodman and Grichting, 1983). The lipolytic effect of GH has been questioned

as being due to lipolytic contaminants in the GH preparations used (Frigeri, 1980), but Goodman and Grichting (1983) found pure GH to stimulate lipolysis. Moreover, biosynthetic GH has been reported to possess lipolytic activity (Goodman, 1984) *in vitro*.

Paradoxically, growth hormone has been shown to also have a marked and rapid-onset insulin-like, anti-lipolytic effect (Goodman, 1970; Birnbaum and Goodman, 1976; Grichting et al., 1983). The insulin-like effects were seen in hypophysectomized rats or in anti-GH treated animals (Goodman, 1970; Ahrén et al., 1976; Birnbaum and Goodman, 1976; Schwartz, 1980; Grichting and Goodman, 1981). Freshly prepared adipocytes from normal rats were not responsive but preincubation of adipocytes from such rats for 3 h rendered the fat cells responsive to GH in terms of glucose oxidation and lipogenesis (Edén et al., 1982), or the anti-lipolytic effect (Grichting et al., 1983). The general condition, which seems to be required for these insulin-like effects to occur, is that GH has to be suppressed for some hours, indicating that normal secretion of GH to the plasma usually prevents the insulin-like effect.

The similarity of the anti-lipolytic effect of GH to that insulin and the methodology available prompted us to do the study reported in paper IV. Freshly prepared adipocytes from normal rats were insensitive to GH after noradrenaline-stimulation of lipolysis. However, after 2-3 h of preincubation in the storage medium the fat cells changed to a GH-sensitive state and the hormone inhibited noradrenaline-stimulated lipolysis in an insulin-like manner, as earlier had been described with regard to glucose utilization (Edén et al., 1982). The noradrenaline-stimulated increase in activity and phosphorylation of HSL in rat adipocytes preincubated for 3 h was reversed to basal levels by GH, indicating that the hormone specifically caused net dephosphorylation of the regulatory site serine residue in HSL, without any effect on the basal site serine (cf. Strålfors et al., 1984). This work (IV) is the first study to show that GH can act by changing the activity of a target enzyme (HSL) by covalent modification, i.e. a change of the phosphorylation state.

The effects of GH on the time-course and on the extent of phosphorylation and activity of HSL were notably similar to those of insulin (cf. Nilsson et al., 1980). These two hormones may therefore transmit their signals at least partly, through the same mechanism(s). This is supported also by other findings, since GH has been demonstrated, besides the inhibition of catecholamine-induced lipolysis, to reduce catecholamine-stimulated cyclic AMP concentrations and cAMP-PrK activity, to activate low K_m phosphodiesterase and stimulate glucose metabolism (for reviews, see Strålfors and Bel-frage, 1984; Isaksson et al., 1984). GH is the only polypeptide hormone besides insulin itself, that has been demonstrated to have an insulin-like, anti-lipolytic effect. Further studies on the mode of its anti-lipolytic action are therefore of great interest, both in themselves and with regard to the question of the mechanism of the anti-lipolytic effect of insulin.

The refractoriness to GH may be due to a sustained hormone receptor occupation by endogenously formed GH. This is supported by the finding that small doses of GH (3 ng/ml) reinduced the refractoriness of GH after appearance of the insulin-like effects (Grichting et al., 1983). However, after secretion of GH has been suppressed, as by stress, starvation, exercise (Reichlin, 1974) pulsatile GH release may very well cause the insulin-like action in mammals.

MODE OF ANTI-LIPOLYTIC ACTION OF NICOTINIC ACID (V)

Nicotinic acid belongs to the group of water-soluble B-vitamins. Early findings indicated that plasma cholesterol, total lipids and esterified fatty acids decreased during nicotinic acid treatment (Atischul et al., 1955; Gurian and Adlersburg, 1959). Carlson and Orö (1962) showed that nicotinic acid lowered catecholamine-induced levels of plasma FFA in vivo, apparently due to a direct inhibitory effect on FFA release from the adipose tissue (Carlson, 1963). Nicotinic acid inhibits both glycerol and fatty acid release and it is generally assumed that its effects are mediated through a decrease of adenylate cyclase activity (Skidmore et al., 1971; D'Costa et al., 1979; Aktories et al., 1980) and cyclic AMP levels

(Butcher et al., 1968; Wieser and Fain, 1975; D'Costa et al., 1979), implicating that the drug reverses cyclic AMP-induced phosphorylation of HSL.

In paper V we have examined this question by comparing the effect of nicotinic acid on catecholamine-stimulated lipolysis with that on the phosphorylation state of HSL, in isolated rat adipocytes. No measurable decrease of the extent of HSL phosphorylation was found by 100 μ M nicotinic acid, in spite of the fact that the drug caused a 40-60% inhibition of lipolysis. The HSL activity against emulsified lipid substrates was not affected by the drug itself. The response differed from that to insulin and propranolol, both in terms of time-course and extent of the decrease of lipolysis. Most of the anti-lipolytic effect of nicotinic acid was prevented in adipocytes, which had been preincubated for 15 min with 100 μ M ascorbic acid before the addition of noradrenaline.

As discussed in paper V the observed effects of nicotinic acid were clearly cAMP-independent and indirect, as the drug did not have any effect on the activity of the isolated lipase or involved a change of the 32 P-HSL-phosphorylation. It is possible that the effects could be mediated through a change of the intracellular redox state and thereby the glutathione redox system (cf. Davies and Souness, 1981), since ascorbic acid, known to be a reducing agent, prevented the anti-lipolytic effect of nicotinic acid. Moreover, noradrenaline-stimulated lipolysis has been shown to be enhanced by ascorbic acid (Hynie et al., 1970) and many oxidizing agents inhibit lipolysis in adipocytes (Czech, 1977). As nicotinic acid is a precursor of nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP^+), this drug could change the redox state in the fat cells by a raised NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ ratio.

Another possibility, discussed in paper V, which cannot be excluded, is that a decrease of intracellular pH might have taken place, since several acids inhibit lipolysis (Gerritsen et al., 1969; cf. Davies, 1973; Nilsson and Belfrage, 1978; Nilsson, 1981).

and since acidosis is known to inhibit lipolysis (Hjemdahl and Fredholm, 1976; Fredholm and Hjemdahl, 1976b).

It is, as mentioned, generally assumed that nicotinic acid exerts its anti-lipolytic effect through an inhibition of adenylate cyclase activity and reduction of cyclic AMP levels (Butcher et al., 1968; Skidmore et al., 1971; Aktories et al., 1980; Schimmel et al., 1981). However, these observations have been made under conditions which prevent the effects of endogenous adenosine (e.g. by the use of methyl xanthines or adenosine deaminase). It is possible that nicotinic acid does not affect adenylate cyclase when the inhibitory guanyl nucleotide-binding protein (N_1), (Rodbell, 1980), is activated by adenosine (cf. Schimmel et al., 1981). The experimental conditions employed in paper V might be more relevant for the mode of action(s) of the drug in vivo.

SUMMARY

This thesis deals with the short-term regulation of adipose tissue lipolysis. The actions of some lipolytic and anti-lipolytic hormones and drugs in isolated adipocytes were investigated.

A modified collagenase digestion procedure for the preparation of hamster adipocytes has been developed (I). Hamster adipocytes prepared according to this method were more sensitive to lipolytic and anti-lipolytic agents than cells obtained with other, routinely used procedures and the adipocytes seemed to better retain intact plasma membrane receptor structures. Using these fat cells the anti-lipolytic effect was shown to be almost 8-fold more sensitive to insulin concentration than glucose uptake and utilization (I), indicating differences in the mode of insulin signal transmission for these processes. Using the same hamster adipocytes the lipolytic β -adrenoceptors in fat cells were characterised (II). Both β_1 - and β_2 -receptors stimulating lipolysis were found in the hamster adipocytes with a predominance of the response to β_2 -adrenergic stimulation.

ACTH, glucagon and two hog pituitary lipolytic polypeptide fractions were found to stimulate lipolysis in rat adipocytes through cyclic AMP-mediated phosphorylation of the regulatory phosphorylation site in HSL with subsequent enhancement of the activity of the enzyme (III) as shown before for the lipolytic β -adrenergic action. Although this has generally been assumed to be the mode of action of ACTH and glucagon direct experimental proof has been lacking.

Growth hormone exerts anti-lipolytic, insulin-like effects under certain conditions. Freshly made adipocytes, unless from hypophysectomized rats, are not responsive to GH, but 3 h preincubation rendered such fat cells sensitive to the anti-lipolytic action of GH (IV). The hormone caused this effect by a net decrease of the noradrenaline-stimulated phosphorylation and thereby reduction of the activity of HSL, notably similar in several aspects to the corresponding effect of insulin. These findings indicate that GH and insulin might share a common signal chain to the target enzyme, HSL, with integration at an early post-receptor site. This is the first observation that GH can modulate the activity of a target enzyme by covalent modification, in the form of a phosphorylation change.

Nicotinic acid has well-known anti-lipolytic effects and is used as a hypolipidaemic drug. It was found to decrease noradrenaline-stimulated lipolysis in rat adipocytes without a concomitantly decreased phosphorylation of HSL (V). This effect of nicotinic acid was therefore apparently unrelated to any changes of cellular cyclic AMP levels; moreover, no direct effect on isolated HSL by the drug was found. It is suggested that the observed anti-lipolytic action of nicotinic acid might have been mediated through changes of the intracellular redox state, leading to oxidation of functional SH-groups on the lipase. This is supported by the finding that ascorbic acid could prevent the action of the drug. However, if the experimental conditions, although unlike those in vivo, protected an endogenous adenosine inhibition of adenylate cyclase, the inhibition of this enzyme by nicotinic acid might be more important.

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EFFECTS OF INSULIN ON LIPOLYSIS AND LIPOGENESIS IN HAMSTER WHITE ADIPOCYTES WITH HIGH SENSITIVITY TO HORMONES

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A modified procedure for preparation of hamster adipocytes by collagenase digestion under carefully controlled conditions has been developed. The adipocytes were 4- to 8-fold more sensitive to catecholamine stimulation of lipolysis than cells prepared by a commonly used method (Hittelman, K.J., Wu, C.F. and Butcher, R.W. (1973) *Biochim. Biophys. Acta* 304, 188–196) and also more sensitive to the anti-lipolytic action of insulin. The effects of insulin on lipogenesis, measured as [^3H]glucose conversion to cell lipids, and on catecholamine-stimulated lipolysis were compared under identical conditions with the same cell batch. Isoprenaline-stimulated lipolysis was found to be half-maximally inhibited by an insulin concentration 8-fold lower than that stimulating lipogenesis to a corresponding extent (half-maximal effects at insulin concentrations of 40 vs. 300 pM). A similar difference was found when cells had been stimulated with adrenaline instead of isoprenaline.

Introduction

Isolated fat cells from the Syrian golden hamster, *Mesocricetus auratus*, like human fat cells, possess both α - and β -adrenergic receptors [1–3] and may therefore be a more generally useful model system than the more commonly employed rat adipocytes. In initial experiments we found that fat cells from the hamster prepared according to a commonly used procedure [1] were often damaged during the preparation and had low sensitivity to lipolytic hormones. Also, as reported originally [3], hormone-stimulated lipolysis was insensitive to insulin inhibition. In this communication we describe a modified preparation procedure by which hamster fat cells with higher hormonal sensitivity are obtained.

Hamster fat cells have been used to study the effects of insulin on glucose utilization and on catecholamine-stimulated lipolysis. The sensitivity of these two processes to insulin is a matter of contro-

versy. Previous work [4,5] has indicated that the anti-lipolytic effect of the hormone occurs at lower concentrations than its effect on glucose utilization. More recently Green and Newsholme [6] and Thomas et al. [7] reported that it affects both processes over the same concentration range. Supporting the conclusions of the former workers [4,5] we have found that lipolysis is indeed more sensitive to insulin than is lipogenesis, when both processes are studied with the same cell batch under precisely the same conditions. Some possible explanations for this discrepancy are discussed.

Methods

Preparation of adipocytes. Adipocytes were prepared from epididymal and perirenal adipose tissue of male golden hamsters (60–75 g, fed a standard chow ad libitum) as described for rat adipocytes [8–10], with some modifications. Adipose tissue (without mincing) was incubated in plastic scintillation vials in modified Krebs-Ringer buffer (1.5 ml per hamster) with 24 mM Hepes (pH 7.4), 3.5% (w/v) bovine

Abbreviation: Hepes, 2-*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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serum albumin, 0.5 mM glucose and 0.5 mg/ml collagenase [10], for 60–90 min at 37°C in a reciprocal shaker (160 strokes/min, stroke length 37 mm). Optimal cell quality was obtained by less than complete digestion of the tissue, therefore the exact time of incubation was decided upon visual inspection after 60 min of incubation. After digestion the Krebs-Ringer-Hepes buffer (see above), but with 1% (w/v) bovine serum albumin (storage medium), was added (5 ml per hamster). All subsequent steps were performed at room temperature. The suspension was filtered twice through double-layered gauze and the cells were isolated by centrifugation (300 X g, 30 s) and washed five times with the storage medium. The adipocyte concentration was determined in a microhematocrit centrifuge as packed cell volume [11] and the cell suspension was diluted with storage medium to 50 μ l packed cell volume per ml. It could then be stored in the plastic vials for up to 4 h at 37°C with gentle shaking. Immediately before the start of an experiment the cells were transferred into fresh medium.

Each hamster yielded 0.7–0.9 g (wet weight) of adipose tissue from which 0.3–0.4 ml packed cell volume of fat cells, with an average cell diameter of 61 ± 1.7 μ m (mean $\pm$ S.E. of three cell batches; mean hamster weight 62 g) was obtained. 1 μ l packed cell volume corresponded to an average of 8600 ± 700 cells (mean $\pm$ S.E. of three cell batches). The intercellular liquid volume, about 1% of packed cell volume [11], was not taken into account in the calculations.

Intactness of cells was evaluated by their microscopic appearance (whole/broken cells, free fat) and by the insulin stimulation of [$3\text{-}^3\text{H}$]glucose conversion to cell lipids (see below). The volume of free fat, a measure of ruptured cells, was $4.5 \pm 1.4\%$ (mean $\pm$ S.E., eight separate cell batches) of the total of packed cell volume plus free fat. A 4- to 7-fold increase of the [$3\text{-}^3\text{H}$]glucose conversion to cell lipids by 690 pM (100 μ U/ml) insulin and half-maximal stimulation of lipolysis with 10–25 nM isoprenaline was used as criteria for functional intactness of the cell batches used.

Hamster adipocytes were also prepared as described by Hittelman et al. [1].

Determination of lipogenesis. Lipogenesis was measured as the conversion of [$3\text{-}^3\text{H}$]glucose over

1 h at 37°C to toluene-extractable adipocyte lipids, according to the method of Moody et al. [12], but with 24 mM Hepes buffer (pH 7.40) instead of bicarbonate. This exchange of buffers does not appreciably change the sensitivity of this parameter to insulin (Gliemann, J., personal communication). The variation coefficient for repeated determinations from the same cell batch was 5%.

Determination of lipolysis. Continuous pH-stat titration of fatty acid release from the fat cells was used to monitor lipolysis in kinetic experiments [9,10]. Incubations were made in modified Krebs-Ringer buffer with low buffering capacity [10] and 1% bovine serum albumin (w/v), pH 7.40 (pH-stat incubation medium). Lipolysis was also determined as glycerol release with an enzymatic fluorimetric method [13], modified for higher sensitivity [14]. The variation coefficient for repeated glycerol release determinations from the same cell batch was 4%.

Materials

Hepes was from Schwartz-Mann, NY, U.S.A.; noradrenaline, adrenaline bitartrate and DL-isoprenaline hydrochloride from Sigma Chemical Co., St. Louis; collagenase (CLS batch No. 49 K 104) from Millipore Corp., Freehold, NJ, U.S.A. and [$3\text{-}^3\text{H}$]glucose (4.2 Ci/mmol) from the Radiochemical Centre, Amersham, U.K. Monocomponent porcine insulin (26.3 U/mg, 1 pM = 0.145 μ U/ml) was a generous gift from Novo, Copenhagen, Denmark. Bovine serum albumin (Cohn Fraction V) from Sigma was dialyzed against distilled water and filtered through an 0.8 μ m filter before use. Catecholamines were stored as a stock solution containing ascorbic acid (1 mg/ml) at -20°C . It was verified that the final concentrations of ascorbic acid obtained in the incubations did not influence the measurements.

Results

Preparation of hamster adipocytes

Factors proven critical for cell quality and/or yield were size of animals, treatment of fat before digestion, batch and concentration of collagenase, and type and time of shaking. No marked difference was found between male and female animals. Hamsters weighing 60–75 g were optimal; smaller animals

TABLE I

EFFECT OF COLLAGENASE CONCENTRATION AND INCUBATION TIME ON THE INSULIN SENSITIVITY OF LIPO-GENESIS

Fat cells were prepared from hamster adipose tissue by incubation with collagenase at the indicated concentrations for various times, as described in Methods. The effect of 690 pM insulin on the conversion of [3-³H]glucose to toluene-extractable lipids in the cells over 1 h at 37°C was then determined. Each value is the mean of three. Values represent increase of ³H in toluene-extractable adipocyte lipids over control without insulin and are expressed as percentages of the maximal increase obtained, 0.65% of added [³H]glucose.

Collagenase concn. (mg/ml)	Incubation time (min):	Increase of ³ H-labeled lipids				
		45	60	90	120	150
0.25		— ^a	36	53	10	7
0.5		73	100	100	35	10
1.7		3	2	0	—	—

^a Yield of cells too low.

(under 55 g) gave too low a yield of cells, while larger animals (over 100 g) provided more cells, but a larger proportion of them was ruptured and they had lower hormone sensitivity. Fine mincing resulted in a large proportion of damaged cells. Each new batch of collagenase was tested before use, since large differences arose, presumably due to a variable presence of proteases. A collagenase concentration of 0.5 mg/ml and

an incubation time of 60–90 min was found to be optimal, when related to cell yield and insulin sensitivity (Table I).

Adipocyte sensitivity to hormones

The fat cells were quite sensitive to hormones, as demonstrated by the effects of catecholamines and insulin on several parameters. The rate of lipolysis,

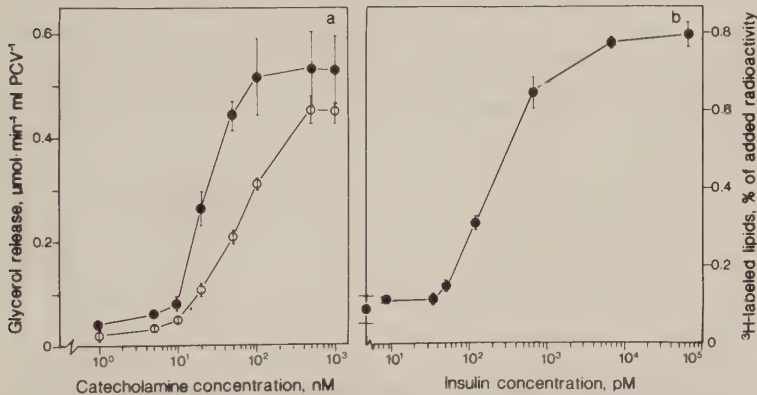


Fig. 1. Sensitivity of hamster adipocytes to hormones. Fat cells (10 μ l packed cell volume per ml) were incubated for 1 h at 37°C in 1 ml modified Krebs-Ringer buffer with 24 mM Hepes (pH 7.40), 1% (w/v) bovine serum albumin, 0.5 mM glucose and other additions as indicated below. (a) Lipolysis rate measured as glycerol release after addition of catecholamines (isoprenaline, $\bullet$ — $\bullet$; noradrenaline, $\circ$ — $\circ$) to the indicated concentrations (each point is the mean $\pm$ S.E. of three experiments with different cell batches; basal lipolysis was 0.018 ± 0.008 μ mol glycerol/min per ml packed cell volume). (b) Lipogenesis measured as the conversion of [3-³H]glucose to toluene-extractable cell lipids after incubation with insulin at the indicated concentrations and 0.1 μ Ci [3-³H]glucose. Each point is the mean $\pm$ S.E. from triplicate incubations in a representative experiment. PCV, packed cell volume.

measured as glycerol release, was half-maximally stimulated by approx. 20 nM isoprenaline or 60 nM noradrenaline (Fig. 1a). The lower sensitivity toward the latter agonist probably reflected the presence of α -adrenergic receptors, which partly inhibit lipolysis [15]. This was also indicated by the finding that 1 μ M phentolamine increased maximally noradrenaline-stimulated lipolysis by 20%. [3 H]Glucose conversion to labeled adipocyte lipids was enhanced up to 9-fold by nanomolar concentrations of insulin (Fig. 1b), with half-maximal effect at 200–300 pM.

Effects of insulin on lipolysis and lipogenesis

Insulin rapidly inhibited the lipolysis induced by an approximately half-maximally stimulating noradrenaline concentration, after a time lag of 2–3 min (Fig. 2a). The anti-lipolytic effect of insulin was dose-dependent, with half-maximal inhibition occurring at approx. 40 pM (Fig. 2b). Thus, lipolysis appeared to be considerably more sensitive to insulin than did lipogenesis (cf. Fig. 1b).

In order to compare directly the insulin effect on

both processes, parallel experiments were performed with the same cell batches, subjecting the cells to precisely the same conditions. To facilitate comparison, the results have been expressed as percentage of maximal effects related to the logarithm of insulin concentration (Fig. 3). Although the data varied considerably between different cell batches, it was quite clear that insulin inhibited maximally isoprenaline-stimulated lipolysis at considerably lower concentration than that at which it stimulated lipogenesis (half-maximal effects at approx. 40 pM compared with 300 pM), representing an almost 8-fold difference in insulin sensitivity. Incubation with isoprenaline in the medium did not have any appreciable effect on the sensitivity of lipogenesis to insulin (compare Fig. 1b with Fig. 3).

Analogous experiments were performed with a maximally stimulating concentration (0.5 μ M) of adrenaline, representing a mixed α - and β -adrenergic agonist. Also in this case was lipolysis more sensitive to inhibition by insulin than was lipogenesis to its stimulation (half-maximal effects at 75 pM and

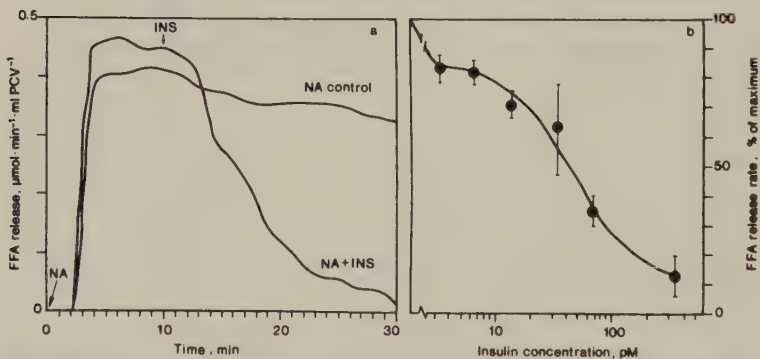


Fig. 2. Effect of insulin on noradrenaline-stimulated lipolysis in hamster adipocytes. Fat cells (10 μ l packed cell volume/ml) were incubated in 10 ml of pH-stat incubation medium with low buffering capacity and 1% (w/v) bovine serum albumin, pH 7.40, at 37°C. The lipolysis rate was measured continuously as free fatty acid (FFA) release by pH-stat titration, after stimulation with 50 nM noradrenaline, in the absence or presence of insulin. (a) Change in lipolysis rate with time after noradrenaline (NA control), or noradrenaline followed after 10 min by 170 pM insulin (NA + INS). Recorder chart from a representative experiment. (b) Effect of various insulin concentrations on noradrenaline-stimulated lipolysis. Insulin was added 10 min after noradrenaline and the free fatty acid (FFA) release rate was measured 10 min later and expressed as percentage of the maximal lipolysis rate. Each point is the mean $\pm$ S.E. of three separate experiments. The curve has been adapted visually to the points. PCV, packed cell volume.

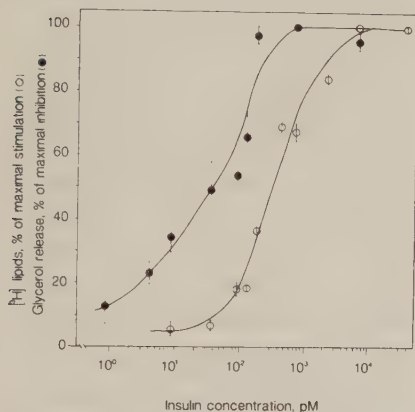


Fig. 3. Effects of insulin on lipolysis and lipogenesis in isoprenaline-stimulated fat cells. Adipocytes were incubated in parallel under precisely the same conditions with the same cell batches, except for the presence of 0.1 μ Ci [3 H]glucose in the glucose conversion experiments. Insulin at the indicated concentrations and 100 nM isoprenaline were added together and the cells (10 μ l packed cell volume/ml) were incubated in the medium described in Fig. 1, for 1 h at 37°C. Each point is the mean $\pm$ S.E. of three experiments with separate cell batches, with incubations performed in duplicates in each experiment. Maximal inhibition of lipolysis was 39.8% and maximal [3 H]glucose conversion to cell lipids was 0.57% of added radioactivity. The curves have been visually adapted to the data points.

300 pM, respectively, calculated as the means from experiments with two separate cell batches).

Discussion

The hamster fat cells prepared as described in this report differed markedly from those isolated according to Hittelman et al. [1]. The combination of fine mincing of the tissue and high collagenase concentration used in that procedure, although convenient in terms of time expenditure, in our hands invariably impaired cell quality and hormone sensitivity. Thus, with such cells 690 pM insulin increased [3 H]-glucose conversion to adipocyte lipids by less than 80% and nanomolar insulin concentration were required to obtain significant inhibition of noradrenaline-stimulated lipolysis (cf. Fig. 3). Isoprenaline

and noradrenaline have been reported to stimulate half-maximally fat cells obtained by that procedure at approx. 160 and 260 nM concentrations, respectively [3], levels which are 4- to 8-fold higher than those required with cells prepared as described above.

The sensitivity of lipogenesis to insulin in the hamster was lower than that reported from experiments with rat adipocytes [5-7,9,12]. Also, catecholamine-stimulated lipolysis was less sensitive to insulin, although the difference was smaller [6,16,17]. In contrast to some recently reported findings with rat adipocytes [6,7] but consistent with other work [4,5] hormone-stimulated lipolysis was found to be several-fold more sensitive to insulin than lipogenesis, when both processes were studied under identical conditions, with the same cell batch. The reason for the discrepancy is not apparent. It was not due to species differences, since also with rat adipocytes we have found lipolysis to be several-fold more sensitive to insulin than lipogenesis, when measured under identical conditions (half-maximal effects at 20-30 pM compared to 80-90 pM [17]). It was not due to the type of adrenergic agonist used, since similar results were obtained from both species with isoprenaline, noradrenaline or adrenaline. However, there are several differences between the present work and that of Thomas et al. [7] and Green and Newsholme [6] which may be important. Thus, lipogenesis and lipolysis were not studied under identical conditions in the work reported in Ref. 7. Cell concentration, incubation times, presence of catecholamines varied, making a direct comparison less valid. Besides, the adipocytes used in that work [7] appeared to be remarkably insensitive to catecholamines, with stimulation being submaximal even at 10 μ M adrenaline, which was the concentration used in all experiments. In spite of this the maximal rate of glycerol release could be calculated to be lower than that obtained by 0.1-0.2 μ M isoprenaline or noradrenaline (Fig. 1a) or in previous work with rat adipocytes in this laboratory [17], or by others [14]. This may indicate that the fat cells used [7] were damaged during the preparation procedure and not comparable to functionally intact cells.

Also in the work of Green and Newsholme [6] a relatively high catecholamine concentration, 1 μ M noradrenaline, was used to stimulate lipolysis. No data regarding sensitivity of this process to catechol-

amine concentration were presented in Ref. 6 but in adipocytes from rats of the same size we have found half-maximal stimulation at approx. 30 nM noradrenaline [14,16]. It is conceivable that micromolar concentrations of catecholamine could decrease the sensitivity of lipolysis to inhibition by insulin and perhaps also increase glucose uptake (cf. Ref. 18). This would offer a possible explanation to the different findings in this report and that of Green and Newsholme [6], which could be tested experimentally.

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CHARACTERISTICS OF THE LIPOLYTIC β -ADRENERGIC RECEPTORS IN HAMSTER ADIPOCYTES

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Hamster adipocyte β -adrenergic receptors were characterized by the effect on the lipolysis rate of several selective β -adrenergic agonists and antagonists. Prenalterol and procaterol, selective β_1 - and β_2 -agonists, respectively, both stimulated the lipolysis rate half-maximally at 1–2 μ M, a concentration approximately 100-fold higher than that needed for half-maximal stimulation by isoprenaline. The maximal procaterol effect was similar to that of isoprenaline, while the effect of prenalterol was 40% lower. Submaximally isoprenaline-stimulated lipolysis was half-maximally inhibited by propranolol at 0.2 μ M and by atenolol and H 35/25, selective β_1 - and β_2 -antagonists, at 23 and 6 μ M concentration, respectively. Highly specific β_1 and β_2 stimulation was induced by incubation with prenalterol or procaterol, with simultaneous maximal selective β_2 or β_1 inhibition, respectively. The maximal β_2 stimulation was approximately twice the corresponding β_1 effect under these conditions, the sum of both effects closely approaching that of maximal nonselective β -adrenergic stimulation with isoprenaline. Similar results were obtained with the selective β_2 -antagonist, ICI 118,551, or the β_1 -antagonists, pamtolol and practolol. The findings are most easily explained by the existence of a heterogeneous β_1 - and β_2 -adrenergic receptor population on hamster adipocytes.

Introduction

Using several different catecholamines Lands et al. [1] classified β -adrenergic receptors into two subtypes, β_1 and β_2 . The lipolytic adrenoceptor on the adipocyte seemed to have similar properties to that of the cardiac receptor (β_1), but differed from those mediating bronchodilatation and vasopressor effects (β_2). Although this classification of the adipocyte β -adrenergic receptor was subsequently supported by findings of others [2–6], recent works have indicated that there are differences between adipocyte and the cardiac β_1 -adrenergic receptors [7–12]. In addition, salbutamol, a selective β_2 -

agonist, has been reported to be an efficient lipolytic agent [6,9] and this was also the case with hexoprenaline, another selective β_2 -agonist [13]. Thus, the classification of the lipolytic β -adrenergic receptor is at present a matter of controversy.

Some of the conflicting findings may reflect species differences since studies have been performed with rat [1,7–11], dog [3,4] and human adipocytes [5,6,9,11,13]. Besides, differences in the preparation of adipocytes may also have to be considered.

In this work, we used suspended isolated hamster adipocytes, prepared with collagenase digestion under such conditions that a high hormonal sensitivity is retained [14], as the experimental system. In contrast to rat fat cells, hamster [15], like human [16], adipocytes contain a large num-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

ber of α - and β -adrenergic receptors and may therefore be a more generally useful model. Lipolysis rate has been measured as glycerol release or as free fatty acid release by a continuous pH-stat titration procedure developed in this laboratory [17].

The results indicate that hamster fat cells contain both β_1 - and β_2 -adrenergic receptors, in analogy with what has been previously found in other cell types [18–20].

Materials and Methods

Materials. Hepes (2-*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was from Schwarz-Mann, NY, U.S.A., DL-isoprenaline hydrochloride from Sigma Chemical Co., St. Louis, U.S.A. and collagenase (CLS batch No. 49K104) from Millipore Corp., Freehold, NJ, U.S.A. Prenalterol hydrochloride (H 133/22), pamtolol sulphate (H 104/08) and H 35/25 (3-isopropylamino-1-(4-(2-methoxyethoxy)phenoxy)propan-2-ol hydrochloride) were generously supplied by Dr. Enar Carlsson of AB Hässle, Mölndal, Sweden. Propranolol hydrochloride, atenolol, practolol and ICI 118,551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) were generously supplied by Dr. Per-Erik Hörnkvist of ICI-Pharma, Gothenburg, Sweden. Procaterol hydrochloride (OPC-2009) was a gift from Otsuka Pharmaceutical Co., Ltd., Osaka, Japan. Salbutamol was a gift from Glaxo Laboratories Ltd., Greenford, U.K. β -Adrenergic agonists were stored as stock solutions with ascorbic acid (1 mg/ml) at -20°C . The final concentrations of ascorbic acid in the incubations did not influence the measurements. Bovine serum albumin (Cohn fraction V) from Sigma was dialyzed extensively against distilled water and filtered through an $0.8\text{-}\mu\text{m}$ filter before use.

Preparation of adipocytes. Adipocytes were prepared as previously described in detail [14]. In short, epididymal and perirenal adipose tissue of male golden hamsters (60–75 g) fed a standard chow ad libitum was treated for 60–90 min in modified Krebs-Ringer buffer with 24 mM Hepes, pH 7.4, 3.5% (w/v) bovine serum albumin, 0.5 mM glucose and 0.5 mg/ml collagenase at 37°C in a reciprocal shaker. The final adipocyte concentration was determined as packed cell volume by centrifuging in a microhematocrit centrifuge and

cell quality evaluated as described [14], according to the method of Moody et al. [22].

The cells could be stored up to 4 h at 37°C with gentle shaking [14]. Immediately before the start of experiment, the cells were transferred into fresh incubation medium [14]. The detailed incubation conditions are given in the figure legends.

Determination of lipolysis. Continuous pH-stat titration of fatty acid release from the fat cells was used to monitor lipolysis in time-course experiments [17]. Incubations were made in modified Krebs-Ringer buffer with low buffering capacity and 1% bovine serum albumin (w/v), pH 7.40 (pH-stat incubation medium) [17]. Lipolysis was also determined as glycerol release with an enzymatic fluorometric method, modified for higher sensitivity [23]. The variation coefficient for repeated glycerol release determinations from the same cell batch was at most 4%.

Data presentation. Time-course effects with lipolysis rate monitored as free fatty acid release were presented as a single experiment, representative of three separate studies with different cell batches. Small differences in the time course between the different experiments precluded averaging of results. In all other cases results are presented as mean $\pm$ S.E. of three experiments with separate cell batches, values from each experiment being based on triplicates. Glycerol release has in all cases been expressed relative to that obtained with the same cell batch by the indicated concentration of isoprenaline (= 100%) in order to enable averaging of data.

Results and Discussion

Time course of lipolysis rate after non-selective β -adrenergic stimulation and inhibition. The basal lipolysis rate (under $0.03\text{ }\mu\text{mol}$ free fatty acids/min per ml packed cell volume) was rapidly increased more than 50-fold by 80 nM of the non-selective β -agonist isoprenaline (Fig. 1), a concentration inducing approx. 80% of maximal lipolytic response (Fig. 2, and see Ref. 14). The isoprenaline-induced lipolysis rate was inhibited rapidly and completely by $25\text{ }\mu\text{M}$ of the non-selective β -antagonist propranolol (Fig. 1). Under the conditions used in the experiment, free fatty acid release rate is closely similar to three times the rate of glycerol release,

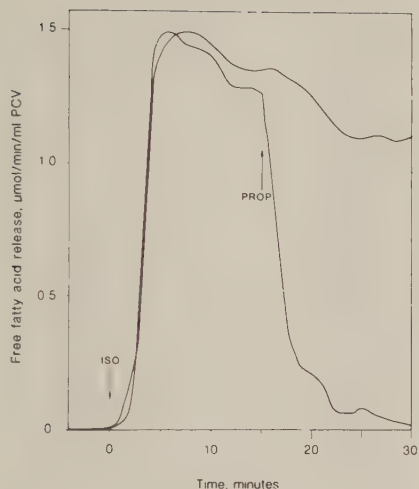


Fig. 1. Time-course of the isoprenaline effect on lipolysis rate and inhibition by propranolol. Fat cells (10 μ l packed cell volume per ml) were incubated in pH-stat incubation medium containing 0.5 mM glucose and 1% (w/v) bovine serum albumin. Isoprenaline (ISO), 80 nM final concentration, was added at the indicated time point and the rate of free fatty acid release, a measure of the lipolysis rate, was followed by continuous pH-stat titration. In a parallel experiment with the same cell batch and under the same conditions propranolol (PROP), 25 μ M, was added 15 min after the isoprenaline, to inhibit the lipolysis. Recorder tracing from representative experiments. PCV, packed cell volume.

indicating that no significant reesterification takes place (data not illustrated). Thus, free fatty acid release is a valid measure of the lipolysis rate (cf. Ref. 17). The maximal value found for this parameter, approx. 0.5 μ mol glycerol/min per ml packed cell volume with 80 nM isoprenaline, is similar to that obtained previously [14].

Effects of selective β -agonists on the lipolysis rate. The fat cell β -adrenergic receptors showed approximately the same sensitivity to a selective β_2 - (procaterol) [24] as to a β_1 -agonist (prenalterol) [25], concentrations for half-maximal effects being in the order of 1–2 μ M for both (Fig. 2). The maximal effect with procaterol was approximately the same as with isoprenaline, while with the selective β_1 -agonist it was 40% less. The adrenoceptor

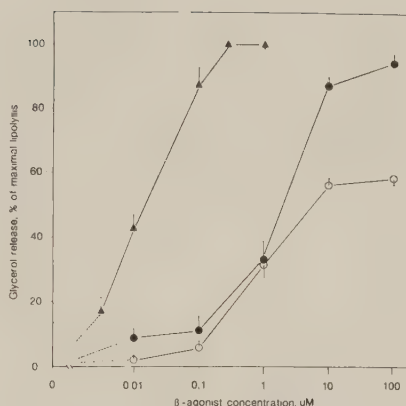


Fig. 2. Effects of various concentrations of selective β_1 - and β_2 -agonists and isoprenaline on the lipolysis rate. Fat cells were incubated as in Fig. 1 but with incubation medium (1.0 ml final volume) containing 24 mM Hepes, pH 7.40, for 1 h in the presence of the indicated concentrations of isoprenaline ($\blacktriangle$), prenalterol ($\circ$), a selective β_1 -agonist, or procaterol ($\bullet$), a selective β_2 -agonist. Lipolysis rate was measured as glycerol release and expressed as percentages of the rate obtained by the maximally stimulating concentration of isoprenaline, 0.4–0.6 μ mol/min per ml packed cell volume. Values are mean $\pm$ S.E. of three experiments with separate cell batches; in each experiment all three agonists were compared simultaneously.

sensitivity to isoprenaline was much higher (80–100-fold, Fig. 2). The isoprenaline sensitivity was similar to previous findings, which also showed that half-maximal noradrenaline stimulation occurred at approx. 60 nM concentration [14]. Additional experiments (data not presented) demonstrated that the selective β_2 -agonist, salbutamol, stimulated lipolysis almost as efficiently as procaterol.

Effects of selective β -antagonists on the lipolysis rate. Submaximally (70%) isoprenaline-stimulated lipolysis was inhibited half-maximally by the selective β_1 -antagonist atenolol at 23 μ M and by the β_2 -antagonist H35/25 at 6 μ M (Fig. 3). The concentrations were at least 30-fold higher than that needed with the non-selective β -antagonist propranolol (Fig. 3). Results with other selective β_1 -antagonists, pamtolol and practolol, and the selective β_2 -antagonist ICI 118,551 [26], lead to the

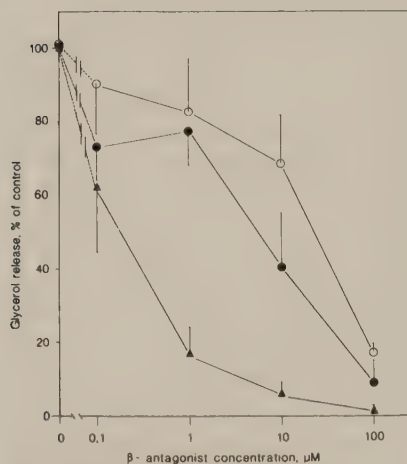


Fig. 3. Effects of various concentrations of selective β_1 - and β_2 -antagonists and propranolol on isoprenaline-induced lipolysis. Fat cells were incubated as in Fig. 2, with 50 nM isoprenaline and the indicated concentrations of the selective β_1 -antagonist atenolol ($\circ$), the β_2 -antagonist, H 35/25 ($\bullet$), and the non-selective β -antagonist propranolol ($\blacktriangle$). Each point is the mean $\pm$ S.E. from three experiments performed with separate cell batches. The data are expressed as percentages of the lipolysis rate obtained with isoprenaline alone (=100%) in each experiment.

same general conclusion: the non-selective β -adrenergic stimulation of lipolysis was inhibited both by β_1 - and β_2 -antagonists, the latter being slightly more potent.

Effects of β_1 - and β_2 -agonists under highly selective conditions. In order to increase the selectivity in the stimulation of the lipolysis with the specific β_1 - and β_2 -agonists used in the experiments described in Fig. 2, fat cells were incubated with maximally stimulating concentrations of these agonists in the presence of highly inhibitory concentrations of the contrary type of antagonist (i.e. β_1 -agonist with β_2 -antagonist and vice versa). The effects on the lipolysis were compared with those of a maximal isoprenaline concentration, and with the maximal propranolol inhibition of this latter effect. Under these highly selective conditions (Fig. 4) maximal β_1 stimulation was approx. half

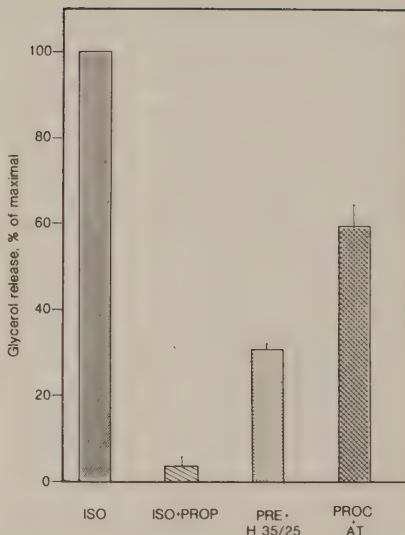


Fig. 4. Effect of highly selective β_1 - and β_2 -adrenergic and isoprenaline stimulation of lipolysis rate. Fat cells were incubated as in Fig. 2 with the following agonists/antagonists: ISO, 1 μ M isoprenaline; ISO+PROP, 1 μ M isoprenaline + 100 μ M propranolol; PRE+H 35/25, 10 μ M prenalterol + 100 μ M H 35/25; PROC+AT, 10 μ M procaterol + 100 μ M atenolol. Each bar is the mean $\pm$ S.E. of three experiments with different cell batches. The data are expressed as percentages of lipolysis rate obtained by isoprenaline alone (=100%) in each experiment.

that of the β_2 stimulation (ratio $\beta_2/\beta_1 = 1.93 \pm 0.16$, mean $\pm$ S.E., $n = 3$) and the sum of both β_1 and β_2 effects was close to that of maximal non-selective (isoprenaline) stimulation ($90 \pm 5.7\%$). These data corroborate those obtained in the experiments illustrated previously (Figs. 2, 3) and demonstrate that lipolysis in hamster adipocytes can be induced both by selective β_1 - and β_2 -adrenergic stimulation. The fact that the same general conclusions were drawn from experiments with several other selective β_1 - and β_2 -adrenergic drugs seems to exclude the possibility that the observed effects would be due to, e.g., intrinsic effects of one or the other of the drugs chosen.

Taken together, the simplest explanation to the

findings is that hamster fat cell β -adrenergic receptors are heterogenous with a predominance of functional β_2 -adrenergic receptors. This heterogeneity is in accordance with findings in dog adipocytes (Ref. 21; Fredholm, B.B., personal communication) and with recent observations in other tissues [20,27,28]. Both types of receptors have also previously been reported to take part in the same biological response [18,19,29,30]. With this interpretation to the results the β -adrenergic receptors in hamster adipocytes would appear to be different from those in rat adipocytes, which have been reported to be homogeneous, with apparently dualistic both β_1 and β_2 characters [12,21]. They would also differ from those in human adipocytes, which have been reported to be mainly of the β_1 -subtype [5,6,11]. However, it has been shown that human adipocyte β -adrenergic receptors differ from the typical cardiac β_1 -adrenergic receptors, e.g., in their sensitivity to cardioselective β -adrenergic blocking agents [9,11]. Thus, it is possible that the β_1 , β_2 -classification, as originally proposed by Lands et al. [1], does not readily apply to the fat cell β -adrenergic receptors [12].

Differences in fat cell preparations may also have been an important factor for classification. We have observed that lipolysis in hamster fat cells prepared with a high collagenase concentration [15] under conditions leading to a more rapid digestion of intercellular material and release of cells could not be induced (under 5% above basal lipolysis) with the β_1 -selective agonist, prenalterol. In addition, cells prepared in this way and stimulated with a submaximal concentration of isoprenaline exhibited a corresponding inhibition pattern. The free fatty acid release rate measured 5 min after addition of the antagonists (1–30 $\mu\text{g}/\text{ml}$) was inhibited as follows, expressed as percent of the stimulated lipolytic rate: H35/25, 100%; ICI 118,551, 74%; propranolol, 100%. Atenolol and pamtalol inhibited less than 5%. Fat cells prepared in this way [15] also have a considerably lower hormonal sensitivity [14] than those used in the present work. Thus, it may well be important to consider cell preparation techniques when attempting to classify the fat cell β -adrenergic receptor.

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ACTIVATION OF ADIPOSE TISSUE LIPOLYSIS BY ACTH, GLUCAGON AND HOG
PITUITARY LIPOLYTIC PEPTIDES THROUGH PHOSPHORYLATION OF
HORMONE-SENSITIVE LIPASE

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Running title: Lipolytic action of polypeptide hormones

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ABBREVIATIONS

cyclic AMP, adenosine cyclic 3',5'-monophosphate; HEPES, 2-N-hydroxy-
ethylpiperazine-N'-2-ethanesulfonic acid; PCV, packed cell volume;
FFA, free fatty acids; SDS, sodium dodecyl sulphate; ACTH, adreno-
corticotrophic hormone; PLF, porcine lipolytic fraction.

SUMMARY

Rat adipocytes were exposed to ACTH¹⁻²⁴, porcine glucagon and certain lipolytically active hog pituitary polypeptide fractions, termed PLF II C and PLF II D (Schleyer, M., Voigt, K.H. and Pfeiffer, E.F. (1976) *Horm. Metab. Res.* 8, 175-181 and 336-340) and the effects on hormone-sensitive lipase phosphorylation and activity examined, to determine their mode of lipolytic action. Both ACTH, glucagon and the hog pituitary polypeptides rapidly induced lipolysis with ED₅₀s in the lower picomolar to nanomolar concentration range. The activation of lipolysis, expressing enhanced hormone-sensitive lipase activity, was preceded by an increase of the extent of phosphorylation of this enzyme. Comparison with the catecholamine effects on these parameters and the modulation of the effect by insulin strongly indicates that the lipolytic hormones and pituitary polypeptide fractions activated lipolysis through a cyclic AMP, and cyclic AMP-dependent protein kinase mediated phosphorylation of the regulatory phosphorylation site known to control the activity of hormone-sensitive lipase (cf. Strålfors, P., Björgell, P. and Belfrage, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81, 3317-3321).

INTRODUCTION

Although there is little doubt that the lipolytic action of ACTH and glucagon, like the action of β -adrenergic agonists, is mediated through phosphorylation of a regulatory phosphorylation site in the rate-limiting enzyme in the adipocytes, the hormone-sensitive lipase (Belfrage et al., 1984; Strålfors and Belfrage, 1984; Strålfors et al. 1984), this has, in fact, never been shown directly. One purpose of the present study has been to do this.

Another aim has been to compare the mechanism of lipolytic action of the catecholamines and these well-known fast-acting lipolytic polypeptide hormones with that of certain lipolytically active polypeptides which have been isolated from hog pituitaries (Schleyer, 1976a,b; Trah, 1983). These polypeptides, in the M_r range of 5000-6000 by SDS-PAGE, have recently been extensively characterized (Trah, 1983). One reason for examining the lipolytic action of these polypeptides has been their lipolytic potency, in swine adipose tissue equal to or greater than that of ACTH (Schleyer et al., 1976a; Trah, 1983), another the interest of our and other groups in the control of lipolysis in the adipose tissue of this species (Lee et al., 1984; Mersmann et al., 1976; Mersmann, 1984a,b).

MATERIALS AND METHODS

Preparation of adipocytes and incubations. Rat epididymal fat cells were prepared from 120-150 g male Sprague-Dawley rats (Anticimex, Stockholm, Sweden) fed a standard chow (Anticimex, Stockholm, Sweden) and tap water ad libitum. After collagenase (0.5 mg/ml) digestion (Nilsson and Belfrage, 1978) adipocytes were washed and diluted to 50 μ l PCV/ml in Krebs-Ringer-HEPES medium (see below), pH 7.40 at 37°C until used (Nilsson and Belfrage, 1978, 1979). The intactness of the cells was routinely tested by the visual absence of lipid droplets (less than 5% of the cell volume) after centrifugation in a microhematocrite centrifuge and by the ability of insulin to stimulate the conversion of [$3\text{-}^3\text{H}$]glucose (0.55 mM) to cellular lipids (Moody et al., 1974). The cells used attained at least a 10-fold increase with 700 pM insulin and half-maximal effect

at approx. 50 pM insulin. One μl of packed cells was equivalent to 1.6×10^4 cells, with an average diameter of approx. 50 μm . The cell concentration has been expressed as PCV, packed cell volume, per ml incubation medium. Incubations in which fatty acid release was measured at 37°C by pH-stat titration (see below) were performed with a fat cell concentration of 50 μl PCV/ml in a modified Krebs-Ringer medium, pH 7.40, with low buffering capacity: 119 mM NaCl, 4.95 mM KCl, 2.54 mM CaCl_2 , 1.19 mM KH_2PO_4 , 1.19 mM MgSO_4 , 0.55 mM glucose and bovine serum albumin, 3.5% (w/v). In ^{32}P -phosphorylation experiments incubations contained [^{32}P]orthophosphate, 25 $\mu\text{Ci/ml}$, at a final phosphate concentration of 50 μM . For storage, and for measurements of glycerol release (with a fat cell concentration of 10 μl PCV/ml) the Krebs-Ringer medium contained 1.0% (w/v) bovine serum albumin, supplemented with 24 mM HEPES, pH 7.40, to maintain a constant pH.

Determination of lipolysis rate. Both FFA and glycerol release reflects lipolysis since reesterification of released free fatty acid is insignificant under the conditions used (cf. Nilsson et al., 1980). Release of fatty acids from adipocytes was continuously measured through the use of a pH-stat titration apparatus (Nilsson and Belfrage, 1979). Glycerol release into the medium was analyzed by the enzymatic fluorometric method (Laurell and Tibbling, 1966; Nilsson and Belfrage, 1978). Each glycerol value is the mean of three or four incubations. Basal glycerol release, i.e. in the absence of added agonists, has been subtracted.

Determination of the extent of phosphorylation of hormone-sensitive lipase within intact fat cells. The extent of hormone-sensitive lipase phosphorylation was estimated from the incorporation of $^{32}\text{P}_i$ into the $M_r=84000$ phosphoprotein band in SDS-polyacrylamide slab gels, after electrophoresis of delipidated whole fat-cell proteins (Belfrage et al., 1980; Nilsson et al., 1980). At appropriate times quadruplicate samples (0.25 ml) of the fat cell suspension were transferred to 0.4 ml Beckman Microfuge B polyethylene tubes containing 0.1 ml of dinonylphthalate. The fat cells were immediately separated from the medium by flotation (8500 g for 5 s at room temperature) through the oil layer, and 50 μl of a cell-disrupting

SDS-solution (Nilsson et al., 1980), containing 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ antipain and 10 $\mu\text{g/ml}$ pepstatin was quickly added to the cell float. After a few minutes the tubes with their contents were frozen at -20°C , until further processed. The time-points given in the figures refer to that at which the SDS-solution was added to disrupt the cells (within 10 s of the end of the centrifugation). After thawing, the tubes were sliced through the dinonylphthalate layer, the solubilized cell float from two replicate tubes transferred to one conical glass tube, the cell-proteins precipitated in acetone and delipidated by repeated extractions with organic solvents as described in Benjamin and Singer (1975). The dried cell-protein pellet (100-150 μg) was then dissolved in SDS sample solution (Nilsson et al., 1980). Complete dissolution of the cell-proteins, as verified by the absence of ^{32}P -labelled material on the top of the separating gel, was critically dependent on repeated heating of the samples to 96°C , sonication in a sonifying bath and Vortex-mixing over a period of up to 3 hours. SDS-polyacrylamide-gel electrophoresis was performed essentially according to Laemmli (1970) in slab gels (Nilsson et al., 1980). Differences in the load of cell-proteins within each experiment were less than 10% and were not corrected for. After electrophoresis the stained and dried gels were autoradiographed on Osray M3 films (Agfa-Gevaert, Belgium) over 2-3 days. The autoradiographs were scanned with a soft laser densitometer at 633 nm (model SL 504, Biomed Instruments, Chicago, IL). The relative ^{32}P -content was estimated as the peak height of the ^{32}P -HSL-band ($M_r=84,000$) (cf. Nilsson et al., 1980; Björgell et al., 1984). This measure correlated well with ^{32}P -radioactivity determined by scintillation counting of cut-out and dissolved gel slices (cf. Nilsson et al., 1980). Values obtained are the means of two cell-protein samples representing four 0.25 ml samples of the fat cell suspension. The variation coefficient for duplicates was 6-8%.

Expression of results. All values have been related to PCV (Nilsson and Belfrage, 1978). In Fig. 1 pooled data from separate experiments with different cell batches have been expressed as mean $\pm$ SEM ($n=3$

independent experiments). In the other figures data from individual representative experiments have been illustrated.

Polypeptide hormones and lipolytically active hog pituitary polypeptides. ACTH¹⁻²⁴ ($M_r=2934$) was a generous gift from CIBA-GEIGY AG (Basel, Switzerland). Porcine glucagon ($M_r=3485$) and mono-component porcine insulin ($M_r=5734$, essentially glucagon-free, $1 \text{ pM}=0.145 \text{ } \mu\text{U/ml}$) was a generous gift from NOVO Laboratories (Gentofte, Denmark). Hog pituitary polypeptides in the form of two highly purified chromatographic fractions, PLF (porcine lipolytic fraction) II C and II D (these designations will be used in the following), were obtained as previously described (Schleyer et al., 1976a,b). The lipolytically active polypeptide in PLF II C, containing 43 amino acids and with an apparent M_r of approx. 5000, constitutes approx. 30% of the proteins of that fraction (Trah, 1983). In PLF II D the lipolytic activity resides in four structurally very similar polypeptides (separable by reversed-phase HPLC) in the M_r range of 5200-6200 by SDS-PAGE, which together constitute approx. 40% of the PLF II D fraction protein (Trah, 1983). In the calculations of molar concentrations of the respective PLF II C and D polypeptides an $M_r=5000$ and these percentage compositions have been used. The polypeptide hormones and PLF II C and D were dissolved in buffered solutions containing 5 mg/ml of bovine serum albumin (crystalline, Sigma, St. Louis, U.S.A.) as a bulk protein. They were either freshly prepared (PLF II C and D), or stored for up to a few weeks at -20°C (ACTH, glucagon and insulin).

Other materials. [^{32}P]orthophosphate and [$3\text{-}^3\text{H}$]glucose was from the Radiochemical Centre (Amersham, UK); collagenase (type CLS, batch 49K104 or W2C 029) was from Worthington Biochemical Corporation (Freehold, NJ). Bovine serum albumin fraction V, dialyzed extensively against water, was obtained from Sigma (St. Louis, MO) as was dl-propranolol. Acrylamide and bisacrylamide were obtained from Bio-Rad (Richmond, CA). Leupeptin, antipain and pepstatin were from the Peptide Institute (Osaka, Japan). All other chemicals and reagents were of the highest grade commercially available.

RESULTS AND DISCUSSION

Concentration dependencies for the lipolytic action of ACTH, glucagon and hog pituitary polypeptides. As illustrated in Fig. 1 all the lipolytic agents used enhanced the rate of lipolysis in rat adipocytes in the lower nanomolar or picomolar concentration range. Approximate ED_{50} s were 1 pM for ACTH, 3 nM for glucagon, 60 nM for PLF II C and 0.7 nM for PLF II D. Similar maximal lipolysis rates were obtained with each of the lipolytic agents. The very high lipolytic potency of ACTH in adipocytes from young rats is illustrated by these results, in accordance with previous findings (Opmeer et al., 1978; Wong and Loten, 1981; Oelofsen and Ramachandran, 1983). The ED_{50} for glucagon is also similar to what has been reported by others (Sonne and Gliemann, 1977; Heckemeyer et al., 1983). The PLF II D polypeptides are quite potent as lipolytic agents in rat adipocytes, although less so under the present experimental conditions than ACTH. Under other incubation conditions and with bigger fat cells from older animals the PLF II D polypeptides have previously been found to be approximately equally potent as ACTH (Schleyer et al., 1976a; Trah, 1983). The PLF II C polypeptide was considerably less potent in these experiments than the PLF II D polypeptides. However, previous work has shown that the two types of polypeptides were equally potent in swine adipose tissue, with ED_{50} in the 3 nM range (Schleyer et al., 1976a). The reason for this species difference is not known.

Time-course of phosphorylation of hormone-sensitive lipase and activation of lipolysis by ACTH and glucagon. Incubation of rat fat cells with $^{32}P_i$ under the conditions employed in Fig. 2 leads to a rapid formation of $[\gamma\text{-}^{32}P]\text{ATP}$ in the cells and an incorporation of ^{32}P into a serine residue at a "basal" phosphorylation site on hormone-sensitive lipase (Nilsson, et al., 1980; Strålfors et al., 1984). This ^{32}P -phosphorylation (Fig. 2A) represents phosphate turnover at this basal site and does not influence the lipase activity and, thus, has no effect on the lipolysis rate (Fig. 2B). Under the conditions of the experiment re-esterification of released fatty acids is insignificant (Nilsson and Belfrage, 1979; Nilsson et al.,

1980; Fredrikson et al., 1981). FFA release therefore directly reflects the rate of lipolysis.

Exposure of the fat cells to submaximal concentrations of ACTH or glucagon caused a rapid ($t_{1/2} < 1$ min) incorporation of ^{32}P into hormone-sensitive lipase (Fig. 2A) accompanied after a short time lag by a corresponding increase of lipase activity, i.e. the lipolysis rate (Fig. 2B). These results are similar, both qualitatively and quantitatively, to those obtained previously with noradrenaline as the lipolytic agent (Nilsson et al., 1980) and provide direct experimental evidence that ACTH and glucagon induce lipolysis in fat cells by enhancing the phosphorylation of hormone-sensitive lipase. Although the individual phosphorylation sites have not been directly examined in this study, the stimulation of the phosphorylation by the polypeptide hormones is almost certainly due to activation of cyclic AMP-dependent protein kinase and phosphorylation by this enzyme of the serine residue at the regulatory phosphorylation site on hormone-sensitive lipase (Strålfors et al., 1984).

Time-course of phosphorylation of hormone-sensitive lipase and activation of lipolysis by hog pituitary polypeptides. In analogous experiments rat fat cells were exposed to the lipolytically active polypeptides in the PLF II C (Fig. 3) and PLF II D (Fig. 4) preparations. In both cases the polypeptides caused rapid enhancement of the phosphorylation of hormone-sensitive lipase and corresponding stimulation of the lipolysis rate. The supramaximal concentrations of the polypeptide fractions which were used caused more extensive phosphorylation of the lipase and higher lipolysis rates than with ACTH and glucagon, but the results were otherwise similar. Addition of insulin 5 min after the start of the lipolysis activation reversed the increase of the phosphorylation and activity of the lipase. Insulin has previously (Strålfors et al., 1984) been shown to exert its anti-lipolytic effect through net dephosphorylation of the phosphoserine at the regulatory phosphorylation site on hormone-sensitive lipase. This serine residue is phosphorylated by cyclic AMP-dependent protein kinase on the isolated lipase (Strålfors and Belfrage, 1983) as well as in the intact fat cell (Strålfors et al.,

1984). Thus, these findings indicate that also the action of the hog pituitary polypeptides, like that of β -adrenergic agonists, was mediated through a cyclic AMP-dependent protein kinase catalyzed, reversible phosphorylation of hormone-sensitive lipase. As expected, the β -adrenergic antagonist propranolol did not affect the action of the pituitary polypeptides at a concentration which caused 70% inhibition of the lipolytic response to submaximal isoprenaline concentration (Table 1).

Thus, it seems reasonable to conclude that ACTH, glucagon and the lipolytically active polypeptides from hog pituitary examined in this work exert their lipolytic action on rat fat cells through the same mechanism as noradrenaline (Nilsson et al., 1980), i.e. by interaction with specific plasma membrane receptors which transmit the hormonal signal to and activate adenylate cyclase, causing increased cellular cyclic AMP levels, cyclic AMP-dependent protein kinase activation and phosphorylation of the specific serine residue at the regulatory phosphorylation site on the rate-limiting enzyme, the hormone-sensitive lipase.

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Table 1

LACK OF EFFECT OF PROPRANOLOL ON LIPOLYSIS INDUCED BY HOG PITUITARY LIPOLYTIC PEPTIDE FRACTIONS

Adipocytes (10 μ l PCV/ml, 1 ml) were incubated for 60 min, 37°C, in Krebs-Ringer-HEPES medium, as described in Methods. The effects of 1 μ M propranolol on lipolysis induced by PLF II C (18 nM), PLF II D (2.4 nM lipolytically active peptide concentrations) and isoprenaline (80nM) were compared. All compounds were added simultaneously and lipolysis measured as glycerol release. The glycerol release rate for each lipolytic substance was set to 100% in incubations without propranolol. Values are mean $\pm$ SEM of three experiments.

Lipolytic agent	Addition of propranolol
	Glycerol release rate, % of control
Isoprenaline	29.0 $\pm$ 0.7
PLF II C	113.3 $\pm$ 10.0
PLF II D	109.8 $\pm$ 3.7

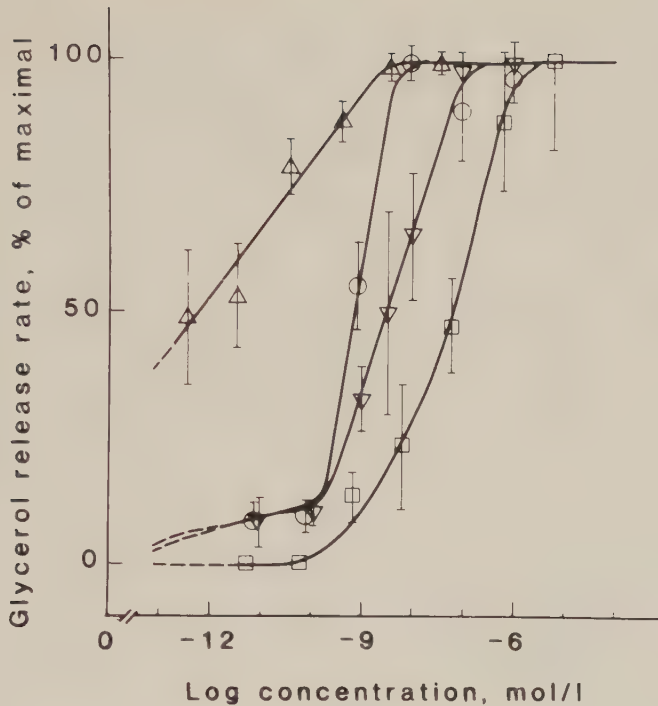


Fig. 1 Concentration dependency for activation of lipolysis by ACTH, glucagon and hog pituitary polypeptides. Rat adipocytes were incubated in Krebs-Ringer medium with 24 mM HEPES, 1% (w/v) bovine serum albumin and 0.55 mM glucose at pH 7.40, 37°C, in the presence of the indicated concentrations of ACTH (Δ), glucagon (∇) and hog pituitary polypeptide fractions PLF II C ($\square$) and PLF II D ($\circ$). The indicated concentrations for PLF II C and PLF II D represent calculated molar concentrations of the lipolytically active polypeptides (see Materials and Methods). Lipolysis was measured as net glycerol release over 1 h and expressed as percentages of the value obtained by the maximally stimulating concentration for each agent. Values are mean $\pm$ SEM of three experiments performed with different cell batches. Maximal lipolysis, expressed as μmol glycerol/min/ml PCV, was 0.52 ± 0.08 for ACTH, 0.39 ± 0.08 for glucagon, 0.62 ± 0.11 for PLF II C and 0.50 ± 0.04 for PLF II D. Basal lipolysis was < 0.02 $\mu\text{mol}/\text{min}/\text{ml}$ PCV.

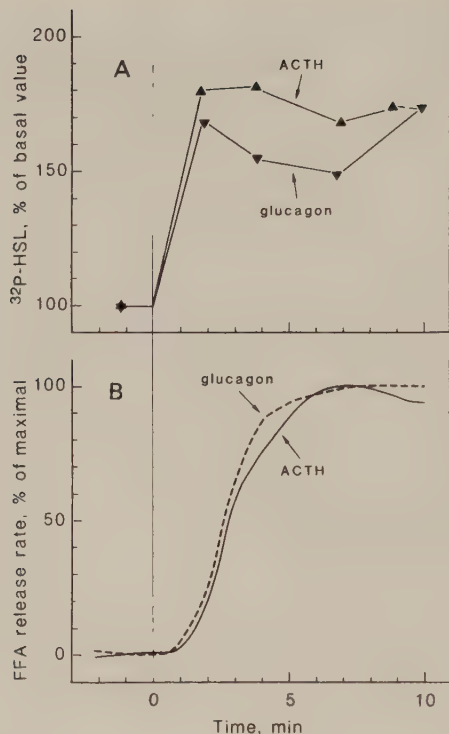


Fig. 2 Effects of ACTH and glucagon on the extent of phosphorylation and activity of hormone-sensitive lipase (HSL). Fat cells (50 μ l PCV/ml) were incubated in modified Krebs-Ringer medium (see Materials and Methods) with 3.5% bovine serum albumin, 0.55 mM glucose and [32 P]orthophosphate (25 μ Ci/ml, final concentration 50 μ M P_i) at pH 7.40, 37°C. **A.** 32 P-HSL was determined as described in Materials and Methods and the extent of phosphorylation related to the "basal" phosphorylation level (=100%) obtained after 40 min preincubation, before additions of hormones. ACTH, $\blacktriangle$, 0.34 nM, or glucagon, $\blacktriangledown$, 100 nM, was added at time zero. Phosphorylation values are the means of four 0.25 ml cell samples. The points indicate the times from the addition of the respective hormone to the addition of disrupting SDS-medium to the samples. **B.** Lipase activity was measured as fatty acid release by continuous pH-stat titration. The curves are adjusted for volume changes. Maximal lipolysis was 0.80 and 1.67 μ mol/min/ml PCV for ACTH and glucagon, respectively. The data are from individual representative experiments performed with different cell batches.

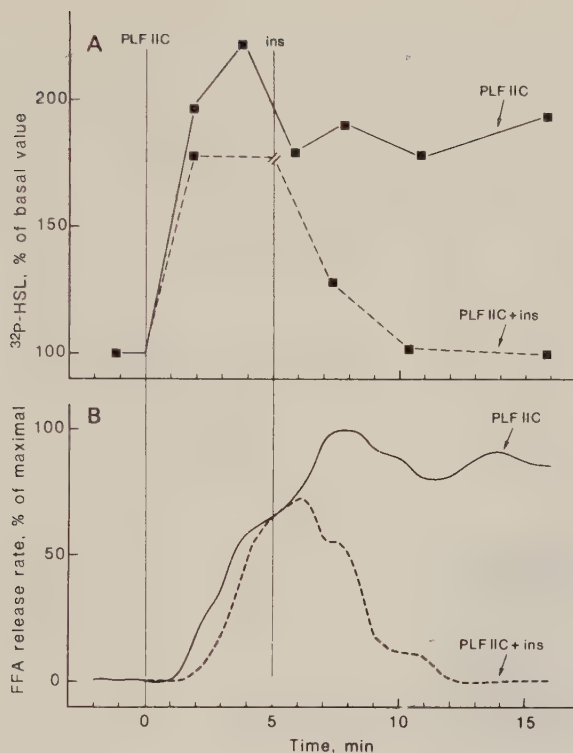


Fig. 3 Effect of hog pituitary polypeptide fraction PLF II C on the extent of phosphorylation and activity of hormone-sensitive lipase (HSL) and modulation of this effect by insulin. Fat cells were incubated as in Fig. 2. A. PLF II C, 5 $\mu\text{g/ml}$ (corresponding to 300 nM lipolytically active polypeptide), alone or followed by 1 nM insulin (as indicated) was added at zero time, 40 min after the addition of [^{32}P]orthophosphate. B. HSL activity was measured as free fatty acid release by continuous pH-stat titration. The curves are adjusted for volume changes. Maximal lipolysis was 3.0 $\mu\text{mol/min/ml}$ PCV with PLF II C alone and 2.3 $\mu\text{mol/min/ml}$ PCV with PLF II C + insulin (maximal lipolysis was not reached before inhibition by insulin). To facilitate comparison values have been expressed as % of maximal lipolysis for the PLF II C curve and the other curve has been assigned the same percentage value at 5 min. The data are from individual representative experiments performed with different cell batches.

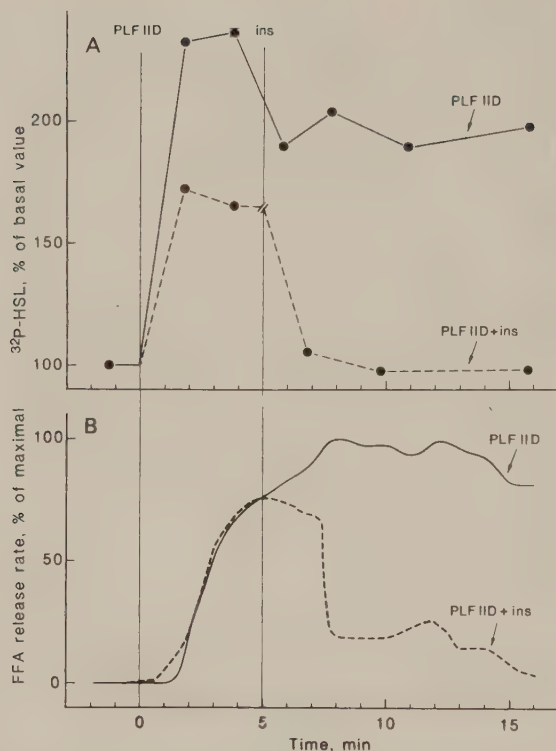


Fig. 4 Effect of hog pituitary polypeptide PLF II D on the extent of phosphorylation and activity of hormone-sensitive lipase (HSL) and modulation of this effect by insulin. Fat cells were incubated as in Fig. 2.

A. PLF II D, 1 $\mu\text{g/ml}$ (corresponding to 80 nM lipolytically active polypeptides), alone, or PLF II D, 8 nM, followed by 1 nM insulin, was added at zero time, 40 min after the addition of [^{32}P]orthophosphate, as indicated.

B. HSL activity was measured as free fatty acid release by continuous pH-stat titration. The curves are adjusted for volume changes. Maximal lipolysis was 3.4 $\mu\text{mol/min/ml}$ PCV with PLF II D alone and 1.32 $\mu\text{mol/min/ml}$ PCV with the lower concentration of PLF II D followed by insulin. The curves have been normalized to facilitate comparison as in Fig. 3. The data are from individual representative experiments performed with different cell batches.

IV

The Antilipolytic, Insulin-Like Effect of Growth Hormone Is Caused by a Net Decrease of Hormone-Sensitive Lipase Phosphorylation*

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ABSTRACT. The mechanism of the antilipolytic effect of GH and the cause of refractoriness to its own action was studied in isolated rat adipocytes. Human GH rapidly inhibited catecholamine-stimulated lipolysis rate, with a time course similar to that of insulin, but only in cells which had been preincubated in the absence of GH for 2–3 h. Half-maximal inhibition was obtained with a GH concentration of 100 ng/ml. Parallel determinations of the lipolysis rate (with a pH-stat titration technique) and the extent of phosphorylation of hormone-sensitive lipase, the rate-controlling enzyme in adipose tissue lipolysis, were made. The extent of lipase phosphorylation, 1.7-fold enhanced by previous noradrenaline stimulation, was rapidly reversed by addition of GH, and the decrease was followed by a parallel decrease in the lipolysis rate. The time course and magnitude of these effects was similar to those obtained with

exposure of the cells to insulin, indicating that the antilipolytic effect of both hormones was exerted through the same mechanism: a net dephosphorylation of the hormone-sensitive lipase.

To study the refractoriness of the fat cells to the action of GH (which was not found with insulin) adipocytes were prepared from hypophysectomized rats 24 h after surgery. With such cells no preincubation was required to obtain the effects of GH on the lipolysis rate and extent of hormone-sensitive lipase phosphorylation. The effects of GH on both of the parameters studied were similar to those obtained in nonhypophysectomized rats. These results suggest that the refractoriness of the fat cells to GH may be explained by a functional inhibition at a site(s) in the series of metabolic events initiated by GH action, which precedes the activation of the hormone-sensitive lipase. (*Endocrinology* 115: 1151–1156, 1984)

IN ADDITION to its obvious importance for the support of normal somatic growth, GH exerts a variety of effects on lipid and carbohydrate metabolism (1, 2). Administration of GH *in vivo* stimulates lipolysis both in normal man and experimental animals. The lipolytic effect of GH is probably the result of a direct effect of GH on adipose tissue since GH *in vitro*, in physiological concentrations, stimulates lipolysis in adipose tissue provided a low concentration of adrenal corticosteroids is also present (2, 3). An important feature of the lipolytic effect of GH is that there is a lag period of approximately 1 h before any release of glycerol and FFA is apparent (3).

Under certain conditions the initial effect of GH is insulin-like, both when GH is administered *in vivo* or added to isolated cells or organs *in vitro*. Thus, GH exerts

transient insulin-like effects in tissues of hypophysectomized rats, from animals injected with antibodies against GH and from young fasted animals (4–6). A common denominator for these animal models is that endogenous GH is eliminated or suppressed, suggesting that normal plasma levels of GH usually prevent the expression of the insulin-like effect of GH.

GH, like insulin, also inhibits the lipolytic effect of catecholamines in adipose tissue of hypophysectomized rats (7). The insulin-like effect of GH is transient and is followed by a time period of refractoriness to GH, *i.e.* the tissue has become normalized, in the sense that GH no longer exerts an insulin-like effect. Goodman and Coiro (8) made the observation that preincubation of adipose tissue from normal rats for a few hours reversed the refractoriness suggesting a rather short half-life for the process(es) maintaining cellular refractoriness to GH. Recently this observation was extended to adipocytes (9). Thus, preincubation of adipocytes from normal rats for 3 h rendered the adipocytes responsive to GH *in vitro* in terms of stimulation of glucose oxidation and incorporation of glucose into triacylglycerols.

The molecular events involved in the insulin-like effects of GH are presently not well understood. Of interest

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for the understanding of the mechanism of GH action are recent studies demonstrating that insulin antagonizes noradrenaline-stimulated lipolysis in adipocytes from normal rats by causing dephosphorylation of hormone-sensitive lipase (HSL) (10, 11), the rate-controlling enzyme in adipose tissue lipolysis (12, 13). Specifically, insulin causes the dephosphorylation of a single serine residue in HSL, termed "regulatory," whose phosphorylation is catalyzed by cAMP-dependent protein kinase and whose phosphorylation state controls the activity of the enzyme (11, 14).

The present study was designed to determine whether GH inhibits noradrenaline-stimulated lipolysis by a similar mechanism, i.e. by a net dephosphorylation of HSL. The effect of GH on HSL activity, i.e. the lipolysis rate, has been measured by continuous pH-stat titration of released fatty acids, in freshly isolated or preincubated adipocytes from normal rats, and in adipocytes from acutely hypophysectomized rats.

Materials and Methods

Animals

Male rats of the Sprague-Dawley strain (AB Anticimex, Stockholm, Sweden), weighing 120–140 g, were used in all experiments. They were housed in a room with artificial lighting from 0530–1700 h; temperature (24°C) and humidity (78%) were held constant. The rats were fed a standard chow (Anticimex) and tap water *ad libitum*. In all experiments fed animals were used.

Two groups of rats were used: 1) normal rats, 120–140 g; 2) hypophysectomized rats, operated at 130–140 g, 24 h before the experiments. Animals were killed by cervical dislocation between 1100 and 1300 h on the day of the experiment. Each adipocyte batch was prepared from adipose tissue of three to five rats.

Preparation of adipocytes and incubations

Rat adipocytes were prepared from the perirenal fat and epididymal fat pads by collagenase (0.5 mg/ml) digestion (15). The packed cell volume (PCV) of the preparation was determined (15) and the cell suspension was subsequently diluted to the final concentration, maximally 50 μ l PCV/ml and stored in Krebs-Ringer-HEPES medium (see below), pH 7.40 at 37°C, until used (15). The cell quality was regularly tested as the ability of insulin to stimulate the conversion of 3-[³H]glucose (0.55 mM glucose, final concentration) to cellular lipids (16). Cells from normal rats attained at least a 10-fold increase with 700 pM insulin, with a half-maximal effect at approximately 50 pM insulin (1-h incubation, 37°C). One microliter of packed cells was equivalent to 1.6×10^4 cells from normal rats (cell diameter $\sim 50 \mu$ m).

Incubations in which fatty acid release was measured by pH-stat titration (see below) were performed in a modified Krebs-Ringer medium, pH 7.40, with low buffering capacity: 119 mM NaCl, 4.95 mM KCl, 2.54 mM CaCl₂, 1.19 mM KH₂PO₄, 1.19

mM MgSO₄, and 0.55 mM glucose. BSA concentration was 1% or 3.5% (wt/vol), as indicated, depending on the fat cell concentration (10 or 50 μ l PCV/ml). In ³²P-phosphorylation experiments incubations contained ³²P-orthophosphate, 25 μ Ci/ml at a final KH₂PO₄ concentration of 50 μ M. Experiments in which lipolysis was measured as glycerol release, and storage, or the 3-h preincubations of cells, were performed in the same incubation media supplemented with 24 mM HEPES, pH 7.40, to maintain a constant pH.

Determination of lipolysis rate

Both FFA and glycerol release reflect lipolysis rate since reesterification of released fatty acids is insignificant under the experimental conditions used (*cf.* Ref. 10). The term "lipolysis rate" will thus be used synonymously for these parameters. FFA release rate from adipocytes was measured continuously by pH-stat titration (17). Rates of lipolysis in controls and in samples with added hormones were simultaneously recorded with two identical pH-stat titration systems (17). The incubation volume used (10–15 ml) allowed multiple sampling for ³²P-hormone-sensitive lipase analysis during lipolysis monitored by pH-stat titration (see below). Values given are corrected for volume changes. Glycerol release into the medium was analyzed by an enzymatic fluorimetric method (15, 18).

Determination of the extent of phosphorylation of hormone-sensitive lipase

The extent of HSL phosphorylation in intact adipocytes was estimated from the ³²P-phosphorylation of the enzyme (as in Ref. 10), but cells were incubated for 55 instead of 40 min before the addition of noradrenaline. At the appropriate times duplicate 0.5-ml samples were taken for ³²P-HSL analysis. Albumin-containing medium was immediately separated from the cells by 5-sec centrifugation at 8,500 $\times g$ in two polyethylene tubes with 100 μ l dinonylphthalate followed by immediate disruption of cells floated in sodium dodecyl sulfate medium (10). The time points indicated in the figures refer to those at which this medium was added to the cells (within 10 sec of the end of the centrifugation). The suspension was then delipidated with organic solvents and the precipitated cell proteins were dissolved in sodium dodecyl sulfate solution. The dissolution was critically dependent upon repeated heating of the samples to 96°C, sonication, and mixing over a 3-h period immediately before the application. Samples were applied to slab gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis the stained and dried gels were autoradiographed with Osray M3 films (Agfa-Gevaert, Belgium) over 3 days. The autoradiographs were scanned with a soft laser densitometer at 633 nm (model SL 504, Biomed Instruments, Chicago, IL). The relative ³²P content was estimated as the peak height of the ³²P-HSL-band (apparent mol wt = 84,000) (see Fig. 3B) above the adjacent baseline on the densitogram. This measure correlated well with ³²P-radioactivity determined by scintillation counting of cut-out and dissolved gel slices. Values obtained from each cell batch are the means of two 0.5-ml samples. The variation coefficient for duplicates was 6–8%.

Expression of results

All values have been related to PCV (15). Unless otherwise indicated pooled data from separate experiments with different cell batches have been expressed as mean $\pm$ SEM (number of independent experiments). Statistical significance has been evaluated with Student's *t* test for paired observations.

Materials

^{32}P -Orthophosphate and 3-[^3H]glucose was obtained from the Radiochemical Centre (Amersham, UK); collagenase (type CLS, batch 49K104) was from Millipore Corporation (Freehold, NJ). BSA fraction V (dialyzed extensively against water) was obtained from Sigma (St. Louis, MO) as was L-noradrenaline bitartrate. Insulin (monocomponent porcine insulin, essentially glucagon-free, $1\text{ pM} = 0.145\text{ }\mu\text{U/ml}$) was a generous gift from Novo Laboratories (Copenhagen, Denmark). Human GH (hGH) was a generous gift from KabiVitrum AB (Stockholm, Sweden). Acrylamide and bisacrylamide were obtained from Bio-Rad (Richmond, CA).

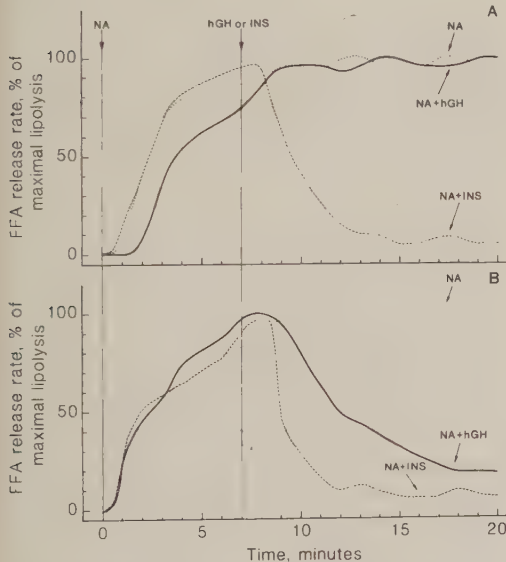


FIG. 1. Time course of effects of hGH and insulin on noradrenaline-induced lipolysis. Adipocytes ($10\text{ }\mu\text{l}$ PCV/ml) were incubated in modified Krebs-Ringer medium (see *Materials and Methods*) with 1% (wt/vol) BSA and the lipolysis recorded continuously as FFA release by pH-stat titration. Noradrenaline (NA), 100 nM ; hGH, 500 ng/ml , or insulin (INS), 700 pM , were added as indicated to A, freshly isolated adipocytes, B, adipocytes preincubated for 3 h in Krebs-Ringer-HEPES medium. The curves shown are from individual experiments with two cell batches (A and B), each curve being representative of two to three incubations. The values have been expressed as percent of the maximal lipolysis rate obtained after noradrenaline (set to 100%). This rate was $1.09 \pm 0.22\text{ }\mu\text{mol FFA released/min}\cdot\text{ml PCV}$ (mean $\pm$ SEM) in eight experiments with the two cell batches.

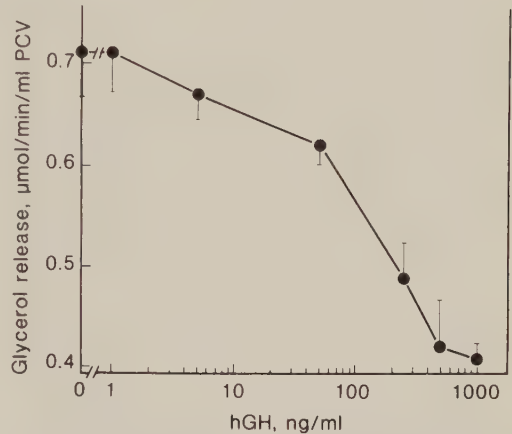


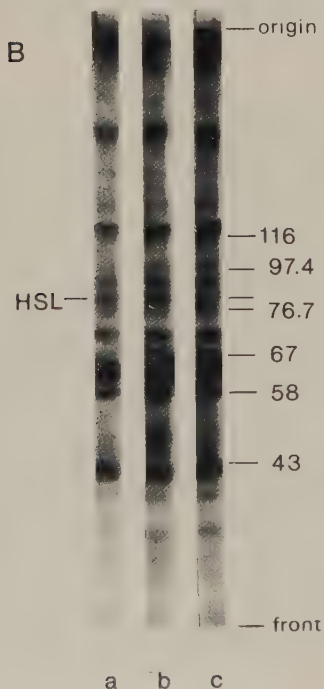
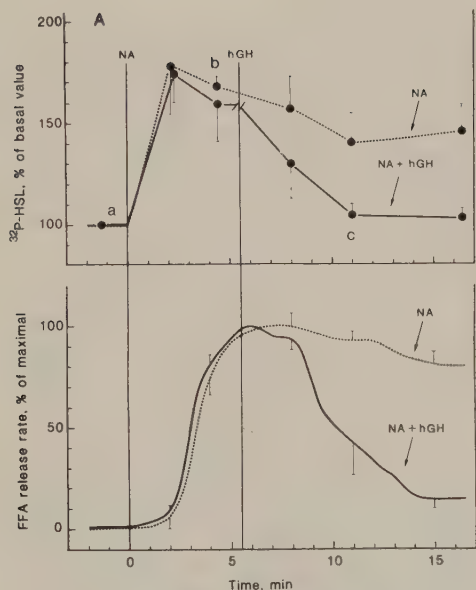
FIG. 2. Concentration dependency of noradrenaline-induced lipolysis to inhibition by hGH. Adipocytes ($10\text{ }\mu\text{l}$ PCV/ml) were preincubated for 3 h as described in Fig. 1. Noradrenaline, 100 nM , plus the indicated concentrations of hGH were then added together to the incubation medium. Lipolysis was measured as glycerol release over the subsequent 60 min. The mean basal lipolysis rate in the absence of added hormones was $0.01\text{ }\mu\text{mol glycerol/min}\cdot\text{ml PCV}$. The indicated values are means $\pm$ SEM from three separate experiments with different cell batches. The values in each experiment are based on triplicate incubations.

Results

The effect of hGH on catecholamine-stimulated lipolysis in freshly isolated rat adipocytes has been illustrated in Fig. 1. Noradrenaline-stimulated (100 nM) lipolysis was not inhibited by 500 ng/ml hGH, in contrast to the marked antilipolytic effect of 700 pM insulin (Fig. 1A). However, when the fat cells had been preincubated for 3 h the noradrenaline-stimulated lipolysis was markedly inhibited by 500 ng/ml hGH (Fig. 1B). Insulin (700 pM) caused a similar effect. A time lag of 2–3 min before an effect was observed was found with hGH, and of 1–2 min with insulin. The lipolysis rate stimulated by 100 nM noradrenaline was decreased by $88.1 \pm 4.6\%$ (mean $\pm$ SEM of three independent experiments with separate cell batches) from its maximal value after the addition of hGH (500 ng/ml).

The concentration dependency of submaximally noradrenaline-stimulated lipolysis to inhibition by hGH in adipocytes preincubated for 3 h was determined (Fig. 2). Glycerol release was decreased by $42.2 \pm 2.1\%$ (mean $\pm$ SEM of three independent experiments), with half-maximal inhibition at a hGH concentration of about 100 ng/ml .

To examine the mechanism(s) by which hGH exerted this antilipolytic, insulin-like effect the extent of phosphorylation of HSL in normal rat adipocytes was deter-



mined in experiments of similar design. Incubation of such fat cells with ^{32}P -orthophosphate for the last 55 min of a 3-h preincubation period caused an incorporation of ^{32}P into HSL to a steady state level at the end of the incubation (Fig. 3, A and B). This ^{32}P -phosphorylation of HSL, which will be referred to as the "basal" HSL phosphorylation, caused no change of the lipolysis rate and has been shown to take place at another phosphorylatable serine residue than the regulatory site residue involved in changes of the function of the enzyme (11, 19). Exposure of the cells to hGH (500 ng/ml) had no significant effect on the extent of phosphorylation of this basal site (100 and 105% of the basal phosphorylation level in two experiments with separate cell batches, each value being the mean of duplicate samples). Addition of noradrenaline (100 nM) to the incubation medium caused a rapid, about 1.7-fold increase in the extent of HSL phosphorylation over the basal level (Fig. 3A, upper panel; 3B) [the average phosphorylation level during the time period of 3–5 min after noradrenaline addition was increased ($P < 0.001$) to $166 \pm 10\%$ (mean $\pm$ SEM) of the basal level in six separate experiments with three different cell batches] followed closely by a corresponding enhancement of the lipolysis rate (*cf.* Ref. 10). Addition of hGH (500 ng/ml) to the incubation medium 5.5 min after the addition of noradrenaline within 5 min decreased the

FIG. 3. Time course of effect hGH on the extent of phosphorylation of HSL and on the lipolysis rate in noradrenaline-stimulated adipocytes. A, Fat cells ($50 \mu\text{l}$ PCV/ml) from normal rats were preincubated in modified Krebs-Ringer medium (see *Materials and Methods*) with 3.5% BSA for 2 h. After change of the medium ^{32}P -orthophosphate ($25 \mu\text{Ci}/\text{ml}$, $50 \mu\text{M}$ phosphate, final concentration) was added and the incubation was continued for another 55 min. In two parallel pH-stat incubations with the same cell batch (see *Materials and Methods*) the effect of addition of submaximally stimulating noradrenaline (NA), 100 nM, was compared with that of noradrenaline followed by hGH, 500 ng/ml, as indicated. The extent of phosphorylation of HSL (^{32}P -HSL) (for determination, see *Materials and Methods*) was related to the basal steady state HSL phosphorylation level (set to 100%) obtained after 55-min incubation with ^{32}P -orthophosphate in the absence of hormones (this level corresponded to 8960 ± 410 dpm $^{32}\text{P}/\text{ml}$ PCV measured by liquid scintillation in six separate experiments with different cell batches). The phosphorylation data points (upper panel) are the means $\pm$ SEM of three experiments with separate cell batches, the values in each experiment based on duplicate samples at each time point. The curves for the FFA release (lower panel) have been calculated by averaging continuous pH-stat titration recordings of this parameter in the corresponding experiments, setting the maximal noradrenaline-induced lipolysis ($2.02 \pm 0.34 \mu\text{mol}/\text{min} \cdot \text{ml}$ PCV, mean $\pm$ SEM, $n = 6$) to 100% in each experiment. Vertical brackets illustrate the SEM ($n = 3$) at the indicated times. B, Autoradiographs of polyacrylamide slab gels after sodium dodecyl sulfate electrophoresis illustrating the adipocyte ^{32}P -phosphoprotein patterns at the time points indicated by a, b, and c in Fig. 3A. HSL, ^{32}P -phosphorylated hormone-sensitive lipase (mol wt, 84,000). Reference proteins: β -galactosidase, 116 kilodaltons; glycogen phosphorylase α , 97.4 kilodaltons; transferrin, 76.7 kilodaltons; BSA, 67 kilodaltons; catalase, 58 kilodaltons; ovalbumin, 43 kilodaltons.

noradrenaline-stimulated extent of HSL phosphorylation to the basal level [$101 \pm 5.5\%$ (mean $\pm$ SEM, $n = 3$) of the basal level, difference not statistically significant. The decrease of the extent of HSL phosphorylation at 5–10 min after hGH addition compared to the level obtained after noradrenaline alone (Fig. 3A, upper panel), was statistically significant ($P < 0.05$). The lipolysis rate decreased by about 85%, closely after the decline of the extent of phosphorylation of HSL, but after a time lag of 2–2.5 min (Fig. 3A, lower panel).

To examine the reason for the refractoriness of the freshly isolated rat adipocytes to the action of hGH the same type of experiments was repeated with fat cells obtained from rats, which had been hypophysectomized 24 h before the experiments (Fig. 4). With these cells hGH exerted a similar effect on the HSL phosphorylation state and lipolysis rate as found in the experiment described in Fig. 3 even without any preincubation.

Discussion

In the present study the time course and the mechanism of the antilipolytic effect of GH has been studied by determining the ability of GH to antagonize the lipolytic effect of noradrenaline in adipocytes from normal and hypophysectomized rats. Measurement of the rate of lipolysis was achieved by continuous recording of the release of fatty acids by pH-stat titration. The results demonstrate that GH causes a dose-dependent inhibition of noradrenaline-stimulated lipolysis in adipocytes from normal rats that have been preincubated for 3 h, but not in freshly isolated adipocytes from normal rats. These results thus confirm previous reports that preincubation renders epididymal fat pads (8) and adipocytes (9, 20) responsive to the insulin-like effect of GH. In adipocytes from hypophysectomized rats, isolated 1 day after surgery, GH exerted an immediate antilipolytic effect in freshly isolated cells. This observation suggests that the lack of antilipolytic effect in freshly isolated adipocytes from normal rats is due to a functional inhibition of this metabolic pathway as a result of the influence of endogenous GH.

The present study gives no information bearing on the mechanism of the ability of endogenous GH to suppress the expression of its own insulin-like activity. Earlier reports have shown that freshly isolated adipocytes from normal rats, that are usually refractory to the insulin-like effect of GH, have a significant number of GH receptors (21, 22), suggesting that refractoriness to the insulin-like effect of GH cannot be explained by depletion of GH-receptors. Edén *et al.* (9) found that the number of specific binding sites for GH increased with time of incubation in adipocytes isolated from normal and hypophysectomized rats, indicating that additional

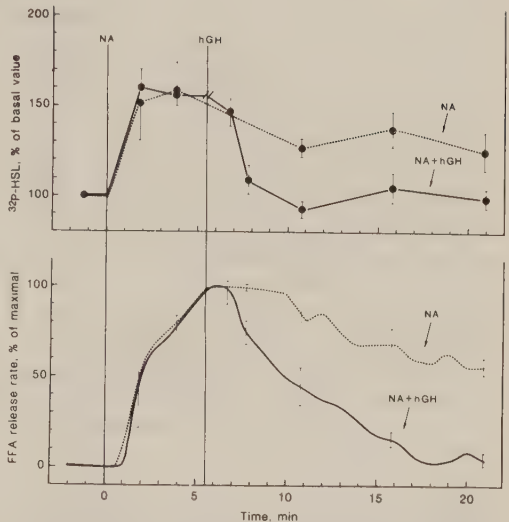


FIG. 4. Time course effect of hGH on the extent of phosphorylation of HSL and on the lipolysis rate in noradrenaline-stimulated adipocytes obtained from acutely hypophysectomized rats. Fat cells, obtained from rats 24 h after surgery were incubated as in Fig. 3, but without any preincubation before addition of ^{32}P -orthophosphate. All other conditions and procedures were as in Fig. 3. The phosphorylation data points (upper panel) are the means and ranges (vertical lines) of two separate experiments with different cell batches, the values from each experiment obtained from duplicate samples. The FFA release curves (lower panel) have been calculated as the mean and range of continuous pH-stat titration recordings from the same experiments. The mean maximal noradrenaline-stimulated lipolysis rate was 1.66 ± 0.26 (mean $\pm$ SEM) $\mu\text{mol FFA}/\text{min} \cdot \text{ml PCV}$ from four experiments with the two cell batches. The basal lipolysis was less than $0.01 \mu\text{mol FFA}/\text{min} \cdot \text{ml PCV}$.

binding sites were developed or unmasked during the incubation period. However, Grichting *et al.* (20) noted that the binding of GH to adipocytes that are unresponsive to the insulin-like effect of GH was indistinguishable from the binding in responsive cells. Thus, the question whether alterations in the number and binding characteristics of GH receptors are involved in the development and maintenance of GH refractoriness needs to be clarified further.

The present results give strong support for the contention that the antilipolytic effect of GH is the result of net dephosphorylation of HSL, as demonstrated by the close temporal and quantitative relationship between these two parameters. The data indicate that GH, as has been shown for insulin (11), specifically causes the dephosphorylation of the regulatory phosphorylatable serine site, with no effect on the basal site in HSL. This finding and the kinetic similarity between the antilipolytic effect of GH and insulin, as found in the present

investigation, suggests that the two hormones, in terms of antilipolysis initiate the same series of metabolic events after receptor binding and activation at the plasma membrane. The finding that insulin, but not GH, inhibited lipolysis in freshly isolated adipocytes from normal rats excludes the possibility that the lack of effect of GH under these conditions is due to a general damage of the adipocyte cell membranes. This possibility is further excluded by the finding that GH exerted insulin-like effects in freshly isolated adipocytes from hypophysectomized rats 1 day after surgery.

The molecular events involved in the insulin-like effects of GH are presently not well understood. There is no apparent structural similarity between the insulin and GH receptor since insulin and GH do not compete for the same binding sites in adipocytes (20). This observation contrasts with the insulin-like growth factors that bind and probably exert some of their metabolic actions via the insulin receptor (23).

It can be concluded from the present study that insulin and GH exert their insulin-like effects through a common biochemical pathway. The nature and the precise cellular location of the process(es) maintaining freshly isolated adipocytes from normal animals refractory to the insulin-like effect of GH remains to be clarified.

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THE ANTI-LIPOLYTIC EFFECT OF NICOTINIC ACID: INHIBITION OF LIPOLYSIS
IN RAT ADIPOCYTES WITHOUT CHANGE OF THE PHOSPHORYLATION OF
HORMONE-SENSITIVE LIPASE

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ABBREVIATIONS

cyclic AMP, adenosine cyclic 3',5'-monophosphate; HEPES, 2-N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PCV, packed cell volume; FFA, free fatty acids; SDS, sodium dodecyl sulphate; N_s , stimulatory guanyl nucleotide-binding protein; N_i , inhibitory guanyl nucleotide-binding protein; IC_{50} , 50% of the maximally inhibitory concentration.

ABSTRACT

Catecholamine-induced lipolysis in rat adipocytes has been inhibited by nicotinic acid and the phosphorylation and activity of hormone-sensitive lipase determined in an attempt to explain the mode of the anti-lipolytic action of the drug. At most, fifty percent inhibition of lipolysis was obtained, with an IC_{50} of approx. $1 \mu M$, without any measurable change of the extent of phosphorylation of hormone-sensitive lipase. The extent and time-course of the inhibition was different from that of insulin and propranolol. Preincubation of the fat cells with ascorbic acid prevented the nicotinic acid effect, but not that of the other anti-lipolytic agents. Nicotinic acid had no direct effect on the activity of isolated hormone-sensitive lipase. It is suggested that the observed effect of the drug, which clearly is indirect and cyclic AMP-independent, could be mediated through change of the intracellular redox state, with oxidation of functional SH-groups in the hormone-sensitive lipase. Alternatively, a decrease of intracellular pH might have taken place. This mode of action of nicotinic acid might differ from that obtained under conditions that prevent inhibition of adenylate cyclase by endogenous adenosine. In the latter case nicotinic acid may be able to exert a more pronounced anti-lipolytic effect by also causing adenylate cyclase inhibition and reduction of cellular cyclic AMP levels with a subsequent decrease of hormone-sensitive lipase phosphorylation.

1. INTRODUCTION

The rate of lipolysis in adipose tissue is determined by the activity of the hormone-sensitive lipase. Hormonal and neural stimuli are believed to regulate the activity of this enzyme through the control of the phosphorylation of a single serine residue at a regulatory phosphorylation site in each $M_r=84000$ subunit (1, for review, see 2). Catecholamines (3) and other fast-acting lipolytic hormones (4) enhance lipolysis by phosphorylating the enzyme through a mechanism involving cyclic AMP and cyclic AMP-dependent protein kinase, while insulin inhibits lipolysis through a net decrease of the extent of phosphorylation of the enzyme, via unknown mechanisms (1, 2).

Nicotinic acid, the well-known hypolipidemic drug, inhibits the rate of adipose tissue lipolysis, in vivo as well as in vitro (5-10). It has been reported that adenylate cyclase activity (6,7) and cyclic AMP levels in fat cells (6,8,9) are lowered by nicotinic acid, but the drug did not affect the activity of the low K_m cAMP phosphodiesterase (7). The mechanism for its anti-lipolytic effect could thus be expected to be simply reversal of cyclic AMP-induced phosphorylation of the hormone-sensitive lipase (3) as with the β -adrenergic antagonist propranolol (1). However, although the sites of the anti-lipolytic action of nicotinic acid, adenosine and the prostaglandins have been assumed to be adjacent to adenylate cyclase (11), it has also been proposed that the drug may exert its action through other mechanisms, e.g. by influencing the intracellular redox state or the activity of the nicotinamide coenzyme oxidases (12). The intracellular redox state could influence the activities of enzymes that are dependent on free SH-groups for their function. This group of enzymes include both adenylate cyclase (13), the low K_m cAMP phosphodiesterase (14), hormone-sensitive lipase (15) and monoacylglycerol lipase (16).

In this work we have therefore examined the mode of the anti-lipolytic action of nicotinic acid by directly relating its effect on the catecholamine-induced lipolysis rate in isolated rat adipo-

cytes, which reflects the activity of the hormone-sensitive lipase, to that on the phosphorylation state of this enzyme in the same cells. No measurable change of the extent of phosphorylation was found in cells in which the drug caused 40-60% inhibition of lipolysis. Thus, at least part of the mode of action of nicotinic acid must be due to other mechanisms than reduction of cellular cyclic AMP and/or cyclic AMP-dependent protein kinase activity.

2. MATERIALS AND METHODS

2.1. Preparation of adipocytes and incubations. Rat epididymal fat cells were prepared from 120-150 g male Sprague-Dawley rats (Anticimex, Stockholm, Sweden) fed a standard chow (Anticimex, Stockholm, Sweden) and tap water ad libitum. After collagenase (0.5 mg/ml) digestion (17) adipocytes were washed and diluted to 50 μ l PCV/ml in Krebs-Ringer-HEPES medium (see below), pH 7.40 at 37°C until used (17-19). The cell concentration has been expressed as PCV, packed cell volume, per ml of incubation medium. The intactness of the cells was routinely tested by the visual absence of free lipid droplets (less than 5% of the cell volume) after centrifugation in a microhematocrite centrifuge, and by the ability of insulin to stimulate the conversion of [3-³H]glucose (0.55 mM) to cellular lipids (20). The cells used attained at least a 10-fold increase with 700 pM insulin and half-maximal effect at approx. 50 pM insulin. One μ l of packed cells was equivalent to 1.6×10^4 cells, with an average diameter of approx. 50 μ m. Incubations in which fatty acid release was measured by pH-stat titration (see below) were performed at 37°C with 50 μ l PCV of fat cells per ml in a modified Krebs-Ringer medium, pH 7.40, with low buffering capacity: 119 mM NaCl, 4.95 mM KCl, 2.54 mM CaCl₂, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄, 0.55 mM glucose and bovine serum albumin 3.5% (w/v). In ³²P-phosphorylation experiments incubations contained ³²P-orthophosphate, 25 μ Ci/ml, at a final phosphate concentration of 50 μ M. For storage, and for measurements of glycerol release with a fat cell concentration of 10 μ l PCV/ml, the Krebs-Ringer medium contained 1.0% (w/v) bovine serum albumin, supplemented with 24 mM HEPES, pH 7.40, to maintain a constant pH.

2.2. Determination of lipolysis rate. Fatty acid or glycerol release rates were used as a measure of the activity of the hormone-sensitive lipase (see below). Since reesterification of released FFA is insignificant under the conditions used (cf. 17,18) both parameters reflect lipolysis. FFA release from adipocytes was continuously measured by pH-stat titration (18,19). Rates of lipolysis in controls and in samples with added hormones and drugs were simultaneously recorded with two identical pH-stat titrators (19). Glycerol release into the medium was analyzed by the enzymatic fluorometric method (17,21). Each glycerol value is the mean $\pm$ SEM of three incubations. Basal glycerol release, i.e. in the absence of added noradrenaline, has been subtracted.

2.3. Determination of the extent of phosphorylation of hormone-sensitive lipase within intact fat cells. The extent of hormone-sensitive lipase phosphorylation was estimated from the incorporation of $^{32}\text{P}_i$ into the $M_r=84000$ phosphoprotein band in SDS/polyacrylamide slab gels, after electrophoresis of delipidated whole fat-cell proteins. At appropriate times quadruplicate samples (0.25 ml) of the fat cell suspension were transferred to 0.4 ml Beckman Microfuge B polyethylene tubes containing 0.1 ml of dinonylphtalate. The fat cells were immediately separated from the medium by flotation (8500 g $\times$ 5 s, at room temperature) through this oil layer, and 50 μl of a cell-disrupting SDS-solution (3), which contained 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ antipain and 10 $\mu\text{g/ml}$ pepstatin, was quickly added to the cell float. After a few minutes the tubes with their contents were frozen at -20°C , until further processed. The time-points given in the figures refer to that at which the SDS-solution was added to disrupt the cells (within 10 s of the end of the centrifugation). After thawing, the tubes were sliced through the dinonylphtalate layer, the solubilized cell float from two replicate tubes transferred to one conical glass tube, the cell-proteins precipitated in acetone and delipidated by repeated extractions with organic solvents as described in (22). The dried cell-protein pellet (100-150 μg) was then dissolved in SDS sample solution (3). Complete dissolution of the cell-proteins, as verified by the absence of ^{32}p -labelled material on the top of the separating gel, was critically

dependent on repeated heating of the samples to 96°C, sonication in a sonifying bath and Vortex-mixing, over a period of up to 3 hours. SDS-polyacrylamide gel electrophoresis was performed essentially according to Laemmli (23) in slab gels (3). Differences in the load of cell-proteins within each experiment were less than 10% and were not corrected for. After electrophoresis the stained and dried gels were autoradiographed on Ostray M3 films (Agfa-Gevaert, Belgium) over 2-3 days. The autoradiographs were scanned with a soft laser densitometer at 633 nm (model SL 504, Biomed Instruments, Chicago, IL). The relative ^{32}P content was estimated as the peak height of the ^{32}P -HSL-band ($M_r=84000$) (cf. 3,24). This measure correlated well with ^{32}P -radioactivity determined by scintillation counting of cut-out and dissolved gel slices (cf. 3). Values obtained are the means of two cell-protein samples representing four 0.25 ml samples of the cell suspension. The variation coefficient for duplicates was 6-8%.

2.4. Enzyme preparations and assays. Rat adipose tissue hormone-sensitive lipase was prepared as a crude pH 5.2 precipitate as described in (15). The enzyme was assayed against emulsified 1(3)-mono[^3H]oleoyl-2-O-oleoylglycerol (a monoester with an ether bond, analogous to di-oleoylglycerol) (25) and trioleoyl[^3H]glycerol (26).

2.5. Materials [^{32}P]orthophosphate and [$3\text{-}^3\text{H}$]glucose was from the Radiochemical Centre (Amersham, UK); collagenase (type CLS, batch 49K104 and W2C 029) was from Millipore Corporation (Freehold, NJ). Bovine serum albumin fraction V, dialyzed extensively against water, was obtained from Sigma (St. Louis, MO) as was dl-propranolol. Monocomponent porcine insulin ($M_r=5734$, essentially glucagon-free, 1 pM=0.145 $\mu\text{U/ml}$) was a generous gift by NOVO Laboratories (Copenhagen, Denmark). Acrylamide and bisacrylamide were obtained from Bio-Rad (Richmond, CA). Nicotinic acid and ascorbic acid was from Apoteksbolaget (Stockholm, Sweden). Leupeptin, antipain and pepstatin were from the Peptide Institute (Osaka, Japan). All other chemicals and reagents were of the highest grade commercially available.

3. RESULTS

The concentration-dependency for the inhibitory effect of nicotinic acid on catecholamine-induced lipolysis has been illustrated in Fig. 1. Maximal inhibition, approx. 50%, was found at 0.1-1 mM nicotinic acid concentration, with half-maximal inhibition at approx. 1 μ M concentration.

The kinetics of the anti-lipolytic effect of nicotinic acid is shown in Fig. 2. Submaximally noradrenaline-induced lipolysis was inhibited approx. 40% by 100 μ M nicotinic acid, the inhibitory effect starting 0-1 min after addition of the compound and being maximal about 12 min after this addition. The effect of nicotinic acid can be compared with the more pronounced effects of insulin (time-lag before start of inhibition, 1-2 min) and propranolol (time-lag, 0-1 min) added at their maximally inhibitory concentrations (Fig. 2). Preincubation of the fat cells with 100 μ M ascorbic acid for 15 min before inducing lipolysis with noradrenaline prevented most of the anti-lipolytic effect of nicotinic acid (Fig. 2), but had no appreciable effect on the decrease of the lipolysis rates caused by insulin or propranolol (data not illustrated).

Nicotinic acid (100 μ M) inhibited submaximally (100 nM) noradrenaline-induced lipolysis (Fig. 3, lower panel) without any measurable change in the extent of phosphorylation of hormone-sensitive lipase, when compared to controls without the drug (Fig. 3, upper panel). Exposure of the fat cells to noradrenaline caused the usual rapid increase of the phosphorylation of hormone-sensitive lipase (Fig. 3, upper panel) due to incorporation of phosphate on the serine residue at the regulatory site (cf. 1). This phosphorylation was accompanied by a marked increase of the lipase activity, reflected in the enhancement of the fatty acid release rate (Fig. 3, lower panel). Approx. 8 min after the addition of noradrenaline the extent of phosphorylation stabilized at about 150% of the basal phosphorylation level. Exposure of the fat cells to nicotinic acid did not change the extent of phosphorylation compared to the control without the drug over the following 10 min, whereas it over the same time period caused an approx. 45% decrease of the rate of lipolysis.

To examine if nicotinic acid in itself had any direct, inhibitory effect on the activity of isolated hormone-sensitive lipase the enzyme was assayed against emulsified lipid substrates in the absence and presence of the drug (0.1 or 100 μ M) (Table 1). No significant effects were found.

4. DISCUSSION

Nicotinic acid inhibited catecholamine-induced lipolysis by maximally 50%, with half-maximal inhibition in the micromolar concentration range, in accordance with other previous studies (5-9,27). The inhibitory effect on glycerol and fatty acid release was similar, indicating that under the present experimental conditions increased re-esterification of fatty acids was not an important part of the effect (cf. 10,28). The response to nicotinic acid was clearly different from that to insulin and propranolol, both quantitatively and in terms of the time-course.

The most significant finding in this study was that nicotinic acid did not alter the extent of hormone-sensitive lipase phosphorylation under conditions during which the enzyme activity, measured as fatty acid release, was significantly depressed by the drug (Fig. 3). Although its anti-lipolytic effect was only about 50% a corresponding decrease of the extent of phosphorylation of hormone-sensitive lipase should easily have been detected, as illustrated by previous work with the anti-lipolytic action of insulin (3), propranolol (1) and growth hormone (24). Another important finding was the selective prevention of the anti-lipolytic effect of nicotinic acid by preincubation of the fat cells with ascorbic acid.

The decreased activity of the hormone-sensitive lipase despite its unchanged extent of phosphorylation points to a site of action adjacent to this enzyme itself, either another type of modification of the enzyme protein or a change of those intracellular conditions which are important for the expression of the activity of the enzyme. However, nicotinic acid in itself did not appreciably affect

the lipase activity against emulsified lipid substrates (Table I). Even if the substrate in the intact fat cell may be different, due to presence of other amphiphilic proteins and lipids on the substrate interface, this finding probably indicates that nicotinic acid interferes with lipase activity in an indirect way rather than directly.

One interesting possibility is that nicotinic acid could reduce the activity of enzymes that are dependent on free SH-groups for their action, by influencing the intracellular redox state and thereby the glutathione redox state (cf. 12). Hormone-sensitive lipase (15), monoacylglycerol lipase (16), adenylate cyclase (13) are all examples of enzymes dependent on free SH-groups for activity. Nicotinic acid is a precursor of nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP^+). A high flux of nicotinic acid into the fat cells could increase the NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ ratio, i.e. change the intracellular redox rate in the oxidized direction. Nicotinic acid could in this way act as an oxidizing agent and inhibit lipolysis and stimulate glucose utilization as has been reported previously for oxidizing agents (for review see 29). Nicotinic acid is known to increase the utilization of glucose in fat cells (30,31). In this context the preventive effect of ascorbic acid on the anti-lipolytic action by nicotinic acid is note-worthy. Ascorbic acid, which is a well-known anti-oxidant (cf. 12), should indeed be expected to have this effect if the mode of anti-lipolytic action of nicotinic acid were as proposed above.

Another possibility, which cannot be excluded, is that nicotinic acid could somehow lower the intracellular pH of the fat cells, with subsequent inhibition of lipolysis (32,33). One factor which might speak for this possibility is the fact that the only other compound which we have found to decrease catecholamine-induced lipolysis without appreciably affecting hormone-sensitive lipase phosphorylation is acetate (17,34). Furthermore, it is known that other short-chain aliphatic acids, lactate and beta-hydroxybutyrate have anti-lipolytic effects. Also, other compounds belonging to the groups pyridines, pyrazoles, isoxazoles and pyrazines inhibit lipolysis,

but only if they contain a free carboxyl group (35). The fact that these compounds are all acids lends some support to, but by no means proves, the hypothesis that they would exert their action through a decrease of intracellular pH.

The anti-lipolytic effect of nicotinic acid, examined in this report, obviously was not primarily caused by reduction of cellular cyclic AMP, which has otherwise been a common finding when fat cells have been exposed to the drug (6,8,9). However, this reduction has generally been observed under conditions that prevented effects of endogenous adenosine, e.g. when methyl xanthines or adenosine deaminase has been used alone (36), or in combination with catecholamines to stimulate lipolysis (7,8,37). Under such conditions the anti-lipolytic effect is much more pronounced than that in this report (7,37) and the IC_{50} is in the lower nanomolar range rather than $1 \mu M$ (38).

In the present study, on the other hand, catecholamine-stimulated lipolysis is only modestly reduced by nicotinic acid, under experimental conditions which would allow endogenous adenosine to accumulate and inhibit adenylate cyclase. This enzyme is believed to be controlled by stimulatory and inhibitory plasma membrane receptors through two regulatory, guanyl nucleotide binding proteins located in the plasma membrane, one mediating stimulatory effects (N_s) and the other inhibitory effects (N_i) (39,40). It is possible that nicotinic acid affects adenylate cyclase only under conditions that prevent endogenous adenosine from inhibiting adenylate cyclase via N_i activation; presence of adenosine may keep N_i in an active state and prevent further activation by nicotinic acid, rendering the fat cells only partly responsive to the drug, as suggested by Schimmel (37). The anti-lipolytic effect of nicotinic acid observed in this study would then represent a cyclic AMP-independent inhibition of N_s -mediated lipolysis, mediated e.g. by one of the mechanisms proposed above. The fact that adenosine is found in the $0.1-1 \mu M$ concentration range in canine adipose tissue (41) and that micromolar concentration of nicotinic acid is required for the anti-lipolytic and lipid-lowering effects in vivo (42) may indicate that the mode

of its action examined in the present investigation may also be relevant for the effect of the drug in vivo.

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TABLE I

Lack of effect of nicotinic acid on the activity of hormone-sensitive lipase.

The enzyme (pH 5.2 precipitate, diluted 20 times) was incubated with indicated concentrations of nicotinic acid for 30 min at 37°C. Values are means $\pm$ SD (n=12), expressed as relative activities towards emulsified 1,3-mono[^3H]oleoyl-2-O-oleoylglycerol (MOME) or trioleoyl[^3H]glycerol (TO) substrates, respectively.

Additions	Substrate	
	MOME	TO
Relative activities, %		
No addition	100.0 $\pm$ 6.1	100.0 $\pm$ 12.6
Nicotinic acid, 0.1 μM	103.6 $\pm$ 5.0	98.6 $\pm$ 13.0
Nicotinic acid, 100 μM	102.8 $\pm$ 7.8	96.8 $\pm$ 11.8

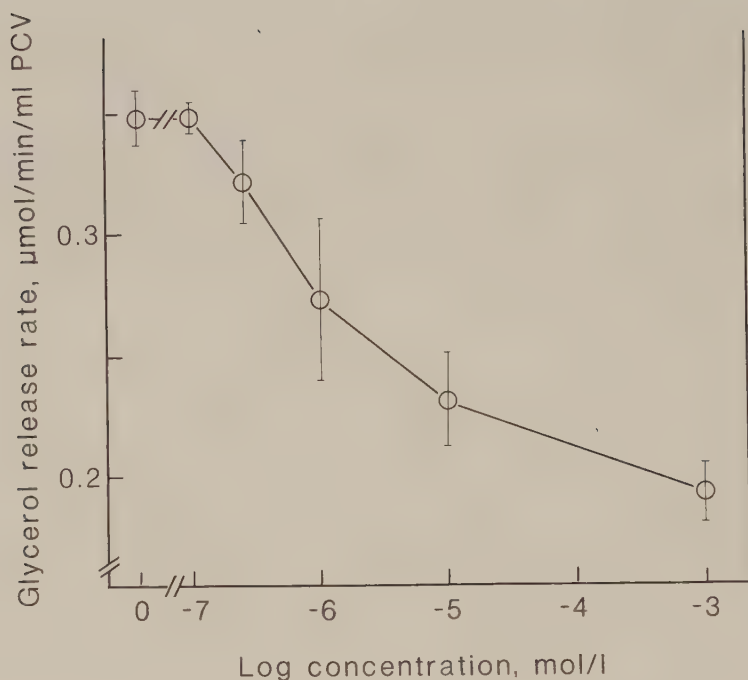


Fig. 1. Dependency of inhibition of noradrenaline-induced lipolysis in isolated rat adipocytes on nicotinic acid concentration. Nicotinic acid and noradrenaline (100 nM) was added together at zero time to 1 ml of a rat fat cell suspension (10 μl PCV/ml) and incubated for 1 h in Krebs-Ringer buffer (see Methods) with 1% (w/v) bovine serum albumin, 0.55 mM glucose, 24 mM HEPES, pH 7.40 at 37°C. Lipolysis was measured as glycerol release relative to that of controls with no added noradrenaline or nicotinic acid. Values are mean $\pm$ SEM, $n=3$ (number of incubations). Basal lipolysis was < 0.01 $\mu\text{mol/min/ml PCV}$.

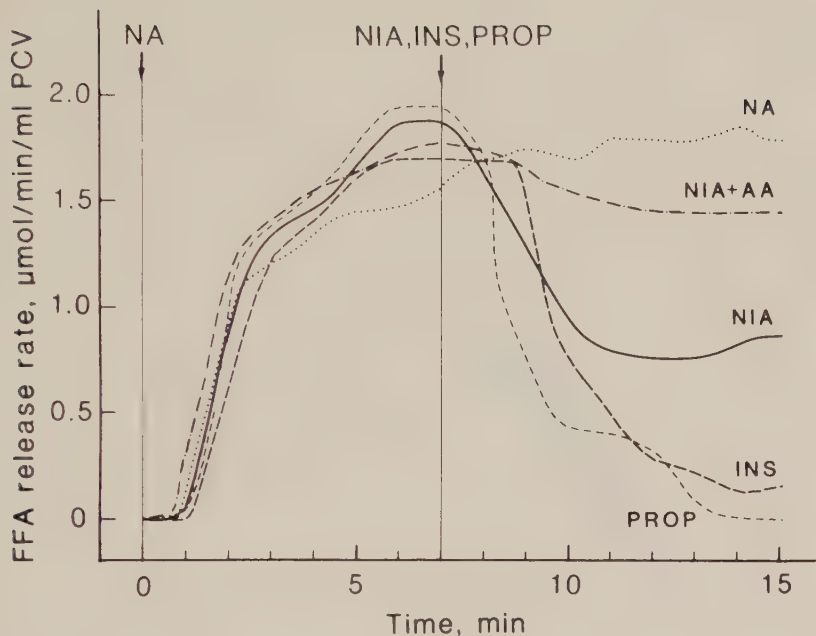


Fig. 2. Comparison of effects of nicotinic acid (with or without 100 μM ascorbic acid), insulin and propranolol on the time-course of noradrenaline-induced lipolysis. Rat fat cells (10 μl PCV/ml, 10 ml) were incubated in Krebs-Ringer medium with 1% (w/v) bovine serum albumin, 0.55 mM glucose, at pH 7.40 (37°C). The rate of the free fatty acid release was followed continuously by pH-stat titration (see Material and Methods). Lipolysis was stimulated by 100 nM noradrenaline (NA). Seven minutes later 100 μM nicotinic acid (NIA), 700 pM insulin (INS) or 10 μM propranolol (PROP) were added. The influence of 100 μM ascorbic acid (AA), added 15 min before noradrenaline, on the anti-lipolytic effect of 100 μM nicotinic acid was also determined (NIA+AA). Recorder tracings from a representative experiment. All incubations were performed with the same cell batch.

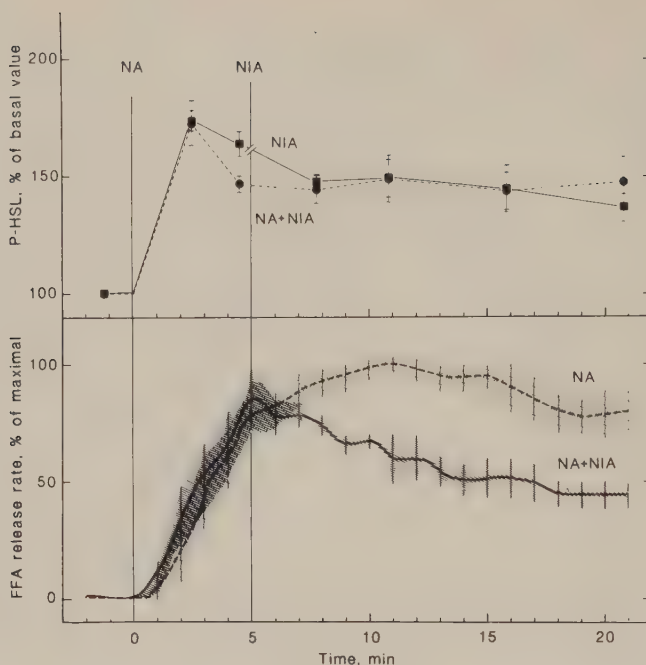


Fig. 3. Effects of nicotinic acid on noradrenaline-induced hormone-sensitive lipase extent of phosphorylation and activity. Rat adipocytes ($50 \mu\text{l}$ PCV/ml, 10 ml) were incubated as in Fig. 2, but with 3.5% (w/v) bovine serum albumin and $50 \mu\text{M}$ sodium orthophosphate combined with carrier-free $[^{32}\text{P}]$ orthophosphate ($25 \mu\text{Ci/ml}$). Noradrenaline (100 nM) was added at zero time (represents 40 min preincubation in the presence of $[^{32}\text{P}]$ orthophosphate) followed by nicotinic acid, $100 \mu\text{M}$, as indicated. The control without nicotinic acid, in a separate incubation vessel, was followed in parallel, under identical conditions. The extent of phosphorylation of HSL (^{32}P -HSL) was related to the basal steady state level of phosphorylation (set to 100%) obtained after the 40 min preincubation with $[^{32}\text{P}]$ orthophosphate. The phosphorylation data points (upper panel) are the means $\pm$ SEM of three experiments with separate cell batches. The curves for the FFA release (lower panel), adjusted for volume changes, have been calculated by averaging continuous pH-stat titration recordings of this parameter in the corresponding experiments, setting the maximal noradrenaline-induced lipolysis to 100% in each experiment. Shaded area around the curves illustrate $\pm$ SEM ($n=3$) at the indicated times. Maximal lipolysis was $1.58 \pm 0.35 \mu\text{mol/min/ml}$ PCV (mean $\pm$ SEM, $n=3$).

